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# ASSOCIATIONS OF PROTEOGLYCANS WITH NORMAL GINGIVAL COLLAGEN FIBRILS IN THE RAT MOLAR PERIODONTIUM

by

(C) JAMES M. PLECASH

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Associations of Proteoglycans with Normal Gingival Collagen Fibrils in the Rat Molar Periodontium submitted by James M. Plecash in partial fulfilment of the requirements for the degree of Master of Science in Oral Pathology.

R. A. McCauley

Supervisor

J. K. Shutka

Date May 12, 1974

To my sons, Ross and Geoffrey.

May this work serve as an inspiration to them
in their future scholastic endeavours.

## ABSTRACT

Electron microscopic studies of the ground substance component of connective tissue obtained from various sites and species have demonstrated definite electron dense morphological patterns of glycosaminoglycan moieties of proteoglycans in close association with collagen fibrils. The purpose of this investigation was to determine if similar morphological patterns of glycosaminoglycans also exist in close association with normal (that is, clinically free of disease) periodontal collagen fibrils in close proximity to the coronal portion of the cementum and the apical portion of the epithelial attachment. The fibrils of concern were those belonging to the dentogingival, the circular, and the alveolar crest principal fiber bundles. A study of this nature is important because knowledge pertaining to glycosaminoglycans of non-pathological periodontal ligament is an essential preliminary in the understanding of the significance of any change in these substances should it occur in pathological situations.

In this study, 1/2 millimeter bucco-lingual sections of tooth and periodontium were obtained from rats fixed and stained by vascular perfusion with glutaraldehyde and ruthenium red. Additional 1/2 millimeter tissue specimens of rat molar periodontal ligament were obtained by excising fresh gingival tissue above the bony alveolus and then stripping the tissue away from the cementum. Then all the tissue specimens were post-fixed by immersion in osmium tetroxide and ruthenium red, according to Luft,(1964, 1965).

Sections cut from perfusion fixed tissue specimens did not exhibit intense electron density except for spherical masses of dense material closely associated with the collagen fibrils. However, thin sections cut from gingival tissue obtained by the second method did demonstrate adequate electron density for study. These electron dense deposits were seen as four forms: interconnecting fine filaments extending approximately at right angles between collagen fibrils; a net-like arrangement of fine filaments in interfibrillar spaces; spherical masses apparently situated at specific minor banding sites within the major periodic 640 Å repeat banding of the collagen fibril; and dense coats surrounding collagen fibrils. Testicular hyaluronidase, papain and saline incubation of tissue specimen blocks followed by exposure of the specimens to the two ruthenium red containing fixatives resulted in a decrease in the thickness of dense coats, partial loss of spherical masses and fine filaments, and complete absence of the filamentous network. It is concluded that these effects after enzyme and saline treatment indicate that the dense coats are probably composed mainly of dermatan sulfate; the spherical masses and fine filaments are probably chondroitin sulfates and/or hyaluronic acid; and the net-like arrangement of fine filaments is probably hyaluronic acid.

However, it is not certain if these observations represent the true morphology of glycosaminoglycans in the native state since cationic dye produces shrinkage of tissue and distortion of fine structure. Furthermore, it is quite likely that aqueous solutions of glutaraldehyde, like formaldehyde, will extract some glycosaminoglycans. Therefore, it is questionable that the electron dense structures seen represent the total amounts of glycosaminoglycans present in interfibrillar spaces.

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# Chapter 1

## THE STRUCTURE AND COMPONENTS OF THE PROBLEM AREA

The periodontium is a term encompassing the gingival epithelium, the alveolar bone, the cementum of the tooth root and the fibrous connective tissue of the periodontal ligament space and the supra-alveolar region (Figure 1). The supra-alveolar connective tissue comprises the mesodermal structures of the gingiva coronal to the crest of the alveolar bone and the periodontal ligament is the fibrous tissue that lies between the root of the tooth and the bony alveolus (Goldman and Cohen, 1973). The main functions of the fibrous connective tissue of these two areas are: to support the tooth in the bony alveolus, to brace the marginal gingiva against the tooth in order to resist forces of mastication, and to unite the free marginal gingiva with the cementum of the root (Glickman, 1972). Like fibrous connective tissue of other anatomical sites, it can be divided into cellular and acellular components. Further subdivisions are possible of the former into cell types and the latter into fiber components and the amorphous ground substance.

The main cell is the fibroblast which, through active protein synthesis, produces the intercellular fibers and most, if not all, of the components of the amorphous ground substance (Leeson and Leeson, 1966). After the fibroblast has made its quota of intercellular substances, it becomes a non-active fibrocyte in the sense that the only

protein synthesis is for intracellular use (Ham, 1965). Other cells normally found within the periodontal fibrous connective tissue include mesenchymal cells, mast cells, and numerous macrophages (Goldman and Cohen, 1973).

The fiber components of the periodontal connective tissue consist of collagen, reticular, elastic and oxytalan fibers. The term fiber with regard to the low power light microscopic appearance of collagen is used to describe straight or slightly wavy threads or ribbons varying between 1 - 12 microns in diameter. At high magnification, the fibers are seen to be made up of a parallel array of several finer threads known as fibrils which measure 0.3 - 0.5 microns in diameter. With the electron microscope (EM), fibrils are shown to be composed of still smaller macromolecular units. These smaller units are generally called microfibrils. They in turn are composed of a parallel, overlapping, aggregation of several tropocollagen molecules, a rod-like structure approximately 2800 Å in length and 15 Å in diameter. The microfibril so formed varies in diameter from 400 - 1000 Å and exhibits a characteristic cross banding about 640 Å apart in thin sections from fixed connective tissue (Ham, 1965; Leeson and Leeson, 1966; Scott and Symons, 1971).

However, Low (1961, 1962) used the term microfibril to describe extracellular fibrous elements much smaller in diameter (40 - 120 Å) than the EM collagen microfibril with the 640 Å periodicity. These ultrastructural units are found in high concentration around elastic fibers and beneath epithelial basement laminae with lesser concentrations among the larger EM collagen microfibrils. They frequently branch and some appear beaded while others resemble flattened tubules

with irregular variations of width. As a result of the beading and the close proximity of microfibrils to fibroblasts in growing tissues, it has been interpreted that this indicates a collagenous nature (Low, 1962; Haust, 1965; Haust and More, 1967; Hashimoto, 1967).

Recently, Vies and Bhatnagar (1970) provided electron microscopic evidence of a "limiting microfibril system" with diameters of 30 - 50 Å which are assumed to be composed of unit assemblies of five collagen molecules as suggested by the geometric model proposed by Smith (1968). When several of these five molecule filaments are packed together with their repeat periods in register, they form the larger unit collagen fibril with the 640 Å cross banding.

Therefore, in this thesis, collagen fibers and fibrils visible with the light microscope will in future be collectively referred to as fibers and the term fibril will be reserved for collagen units measuring 250 - 1000 Å in diameter with a distinct 640 Å periodicity visible in the EM. The term microfibril will then be used exclusively for beaded filamentous structures measuring 40 - 150 Å in diameter, which are assumed to be of a collagenous nature.

The collagen fibers of the periodontal ligament space and supra-alveolar region are organized into two forms according to their histologic appearance. These are the bundles of collagenous fibers which pass from the cementum of the tooth root to the compact bone of the tooth socket, to adjacent teeth, or towards the gingival epithelium surrounding the cervical region of the tooth; and the loosely arranged collagen fibers of the lamina propria beneath the gingival epithelium, plus those fibers surrounding blood vessels and nerves located throughout the fibrous connective tissue. The distinctly orientated collagenous

bundles are referred to as "principle fibers" and in the region described as the supra-alveolar connective tissue, these fiber bundles on the buccal aspect of the tooth (Figure 2), because of their origin, direction and location, are known as (a) the superior fibers of the alveolar crest group; (b) the dentogingival group beginning in the cementum from below the cementum-enamel junction and running out a short distance at right angles to the cementum surface, then fanning out occlusally, horizontally and apically to join with the lamina propria fibers of the free and attached gingiva, as well as the periosteal fibers covering the buccal surface of the alveolar bone; and (c) the circular fiber bundles surrounding the tooth at a level above the crest of the alveolar bone. As the function of the dentogingival and circular fiber bundles is to hold the gingiva tightly against the neck of the tooth, loss of integrity in these fibers will result in the formation of "pockets" between the tooth and gingiva. For convenience, the collagen fiber bundles of the supra-alveolar region will henceforth be collectively referred to as the "gingival periodontal ligament."

Reticular fibers are branching elements of small diameter and probably are young or immature collagenous fibers since, when examined in the EM, they exhibit the same periodicity of the collagen fibril (Leeson and Leeson, 1966; Ham, 1965). In the developed periodontium, they are found beneath the basement lamina in a narrow area adjacent to the epithelium. Reticular fibers are also found investing small blood vessels and nerve fibers (Goldman and Cohen, 1973).

True elastic fibers are present only in the walls of arterioles and arteries (Scott and Symons, 1971); but oxytalan fibrils, with a diameter of approximately 100 Å (Fullmer and Lillie, 1958), are

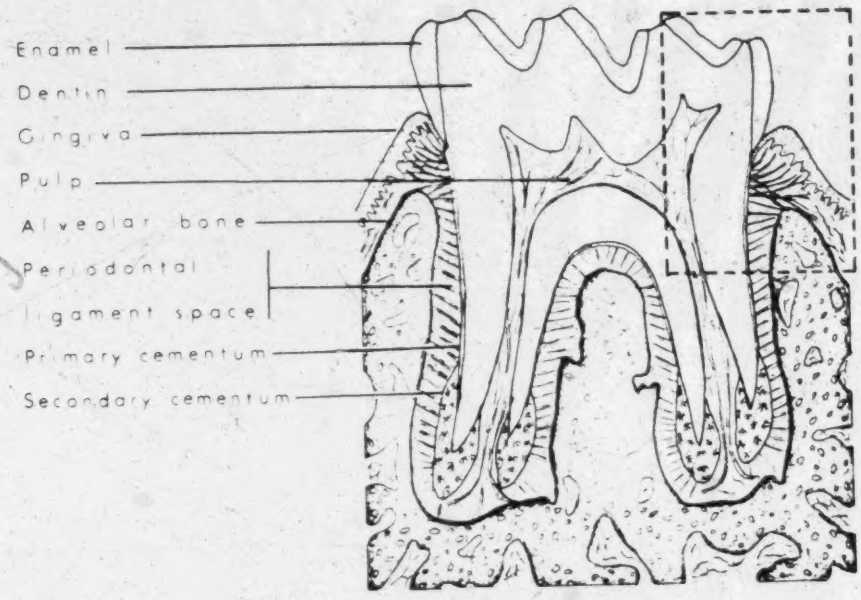



Figure 1. The Rat Molar. Diagrammatic representation of a bucco-lingual section made through the lower first molar. (Redrawn from Schour and Massler, 1949.)

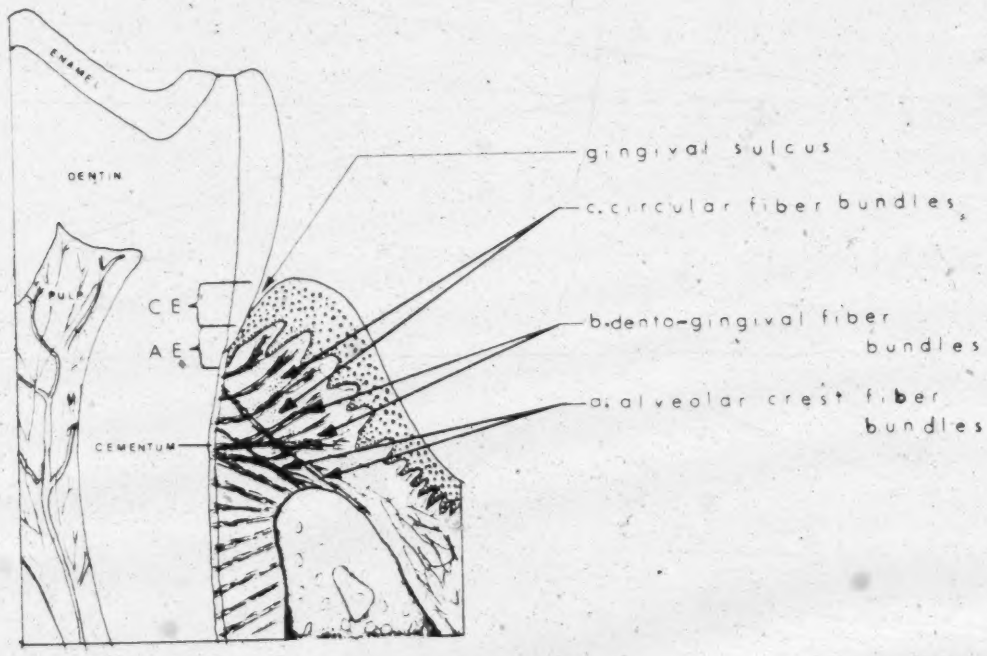



Figure 2. The Supra-Alveolar Periodontal Fiber Bundles of the Buccal Gingiva. Enlarged drawing of area outlined with broken lines in Figure 1.

CE = crevice epithelium
AE = attached epithelium

considered to be a variant of elastic tissue (Fullmer, 1966). Oxytalan fibrils insert into the cementum and extend outward in the same direction as and at various angles to the collagen fibrils; but they do not extend to adjacent teeth or adjacent bone (Fullmer, 1961). Carmichael (1968) observed that the majority of oxytalan fibrils were distributed between blood vessels and either cementum or gingiva, which suggests an anchoring function necessary to compensate for forces of compression likely to create vessel distortion.

The other important constituent of connective tissue, the "amorphous ground substance," evolved from the word "Grundsubstanz" conceived by European histologists in the latter half of the nineteenth century. The original concept of Grundsubstanz, literally meaning "fundamental substance," was a primordium from which cells and fibers of the connective tissue are formed. In contrast, a second term conceived in the same era, the "fiber cement substance," referred to a mucinous medium binding together the formed elements of connective tissue. In later years, with the translation of Grundsubstanz to ground substance, the word was used as a light microscopic or histochemical descriptive term for the matrix in which cells and fibers are embedded (Jackson and Bentley, 1968; Pearce and Grimmer, 1970). With histochemical methods, it is best observed in loose connective tissue as a shapeless area occupying spaces not filled by cells or fibers. Because it is shapeless, it has been called amorphous and has been described as consisting of a sol or gel state, differing from tissue fluids which can be removed by drainage (Schubert and Hamerman, 1968).

The nature of the intercellular matrix or ground substance of loose connective tissue was a controversial subject for many years. Some

investigators believed that the collagenous and elastic fibers were embedded in a jelly-like, amorphous substance somewhat related to the firmer cement substance holding the fibrils together as fibers. The matrix embedded fibers were arranged in lamallae or membranes separated by spaces filled with a minute amount of tissue fluid. Other investigators accepted the lamellar arrangement of fibers, but denied the existence of a fiber embedding amorphous substance. Instead, they claimed the spaces between the fiber lamallae were filled entirely with a liquid colloidal mass, continuous with the tissue fluid (Maximow, 1930).

This debate was resolved by Bensley (1934) who demonstrated that subcutaneous injections of fluid and paramoecia resulted in neither flowing away of fluid nor spread of the organisms as might be expected if there were no barrier of amorphous material present. Using differences in microscopic refractive indices, she observed an interface between the injected fluid and the material of the tissue space surrounding the fluid. She suggested that this "interface" was caused by a viscid substance resembling mucins as indicated by metachromatic staining of the intercellular areas. Thus, it became clear from her observations that there was an amorphous substance, more viscid in nature than tissue fluid, which embedded the cells and fibers of loose connective tissue.

However, the intercellular ground substance of connective tissues in general is far from amorphous in structure, or chemically elementary in composition. Gersh and Catchpole (1960) defined the term ground substance as "a place name for macromolecular aggregates which embrace a number of chemical substances." Investigations with the method of tissue culture (Grossfeld et al., 1957; Matalon and Dorfman, 1970; Shulman and Meyer, 1970) indicated that some of these ground substance components originate from the fibroblast cell. Radioautographic and

histochemical methods which have been well reviewed by Curran (1960), Quintarelli (1965) and Spicer and Henson (1967), have also been of value in determining the location of many of these chemical substances contained within the ground substance. Biochemical investigations (Meyer et al., 1956; Hoffman et al., 1958a), although giving no information about the intercellular location in the connective tissue, have resulted in the identification of the chemical and molecular structure of a large variety of these macromolecules of the ground substance. According to older terminology, those components of the ground substance thus far identified by biochemical techniques were classified (Table 1) into the groups of mucopolysaccharides, glycoproteins and the transient chemical population derived from the blood and local parenchymal cells. The mucopolysaccharides and glycoproteins occur in nature as carbohydrate-protein complexes; however, there has been a great deal of confusion concerning the true meanings of these terms because of the many workers in this field, each of whom has defined these substances in his own way.

For example, the term "mucopolysaccharide" has, at various times, been used to designate a hexosamine containing polysaccharide occurring free or as a chemical derivative of a substance of higher molecular weight (Meyer, 1938, 1945) or as a protien-carbohydrate complex whose reactions are predominantly polysaccharide (Stacey, 1946).

Another term, "mucoprotein," appearing frequently in classifications, has added to the confusion due to its ambiguous usage. In one classification it was synonymous with mucoids which were mucopolysaccharides in firm chemical union with a peptide where the polysaccharide hexosamine content is greater than 4 percent (Meyer, 1945). Mucoproteins were also defined as protein-carbohydrate substances whose reactions are predominantly protein (Stacey, 1946; Kent and Whitehouse,

Table 1. Ground Substance Components of Connective Tissue (from Meyer, 1945; Bettelheim-Jevons, 1958; Gersh and Catchpole, 1960).

I. Mucopolysaccharides

- A. Neutral Mucopolysaccharides (do not contain an acid group)
- B. Acid Mucopolysaccharides
  1. Hyaluronic acid
  2. Chondroitin sulfates A, B and C
  3. Chondroitin
  4. Keratosulfate
  5. Heparitin sulfate
  6. Heparin

II. Glycoproteins

III. Derivatives from Plasma and Local Parenchymal Cells

1. Water and electrolytes
2. Serum proteins including antibodies
3. Enzymes
4. Hormones and vitamins
5. Products of cellular metabolism

1955). Furthermore, mucoproteins have been defined as acid mucopolysaccharide-protein complexes (Meyer, 1953).

In order to arrive at some standardization, Jeanloz (1960) proposed that the prefix "muco" be completely eliminated from the chemical nomenclature and that there be five groups of macromolecular complexes containing carbohydrate components. Only the first three groups will be described, as the fourth and fifth groups belong to the class of glycolipids common to nervous tissue. The first three groups are listed as:

1. pure polysaccharides (e.g., hyaluronic acid, chondroitin sulfate, etc.)
2. polysaccharide-protein complexes where the attachment is a weak salt type linkage (e.g., chondroitin sulfate-protein complex, etc.)
3. glycoproteins defined as carbohydrates attached to a polypeptide by a strong covalent linkage (e.g., mucous secretions, blood group substances, etc.)

The relative ease of separating the components of Group 2 was taken as evidence of the existence of weak links. The elimination of the prefix "muco" was achieved by substituting the terms "glycosaminoglycuronoglycans" for the group known as acid mucopolysaccharides and "glycosaminoglycans" for the amino sugar containing polysaccharides, as ruled by the American Chemical Society Subcommittee on Polysaccharide Nomenclature. This has gained wide acceptance by the majority of workers in this field.

Gottschalk (1962), with his definition of a polysaccharide-protein complex and a glycoprotein, added further enlightenment towards the categorization of these substances. He described the carbohydrate moiety of a polysaccharide-protein complex represented by hyaluronic acid, the chondroitin sulfates, etc., as being composed of heteropolysaccharides characterized by a small regular repeating unit and a high

degree of polymerization. He further states that the carbohydrate of a glycoprotein is of relatively low molecular weight and is composed of one or two or as many as eight hundred heterosaccharides. Each of the heterosaccharides is made up of two or more different sugar residues lacking a short repeating structure and many possess a sialic acid group in a terminal position. In addition, Gottschalk (1966) reported that the distinguishing feature of strong and weak linkages of the Jeanloz (1960) classification is no longer tenable for there is little doubt that covalent linkages are also present between the polysaccharide-protein complexes.

Pearce (1968), taking into account the definition of polysaccharide-protein complexes and glycoproteins proposed by Gottschalk (1962), modified mainly the polysaccharide class (Group 1) of Jeanloz' scheme. The general name of "glycans" was given to this division and subdivisions were created based on the number and types of monosaccharide components. Included in the subdivision of heteroglycans (two or more monosaccharide components) were the glycosaminoglycuronoglycans (e.g., hyaluronic acid) and the glycosaminoglycans (e.g., keratosulfate and sialoglycans). The term "glycosaminoglycuronoglycans" aptly describes a polysaccharide with amino sugars and uronic acid components. However, it is unwieldly to use. In addition, albeit this classification is concise, it is felt that such elaboration is not necessary for this study because many of the various subdivision macromolecular components are not present in fibrous connective tissue. Therefore, as keratosulfate is the only connective tissue member of the glycosaminoglycan designation, the suggested terminology of Balazs (1970) will be adopted in this thesis (Table 2).

Hence, glycosaminoglycans will be the group name for the

Table 2. A Revised Classification of Ground Substance Components (Based on terminology from Balazs, 1970).

1. Glycoproteins (GP)
2. Neutral Heteroglycans (NH) for neutral mucopolysaccharides
3. Glycosaminoglycans (GAG) for acid mucopolysaccharides
   a. Hyaluronic acid (HA)
   b. Chondroitin (Ch)
   c. Chondroitin 4-sulfate (Ch 4-S) for chondroitin sulfate A
   d. Chondroitin 6-sulfate (Ch 6-S) for chondroitin sulfate C
   e. Dermatan sulfate (DS) for chondroitin sulfate B
   f. Keratan sulfate (KS) for keratosulfate
   g. Heparan sulfate (HS) for heparitin sulfate
   h. Heparin (H)
4. Proteoglycans (PG) for polysaccharide-protein complexes
   a. Proteohyaluronic acid
   b. Proteochondroitin 4-sulfate (PCh 4-S)
   c. Proteochondroitin 6-sulfate (PCh 6-S)
   d. Proteodermatan sulfate (PDS)
   e. Proteokeratan sulfate (PKS)
   f. Proteoheparan sulfate (PHS)
   g. Proteoheparin (PH)

chemically isolated acid mucopolysaccharides. Specific reference to members of the group will be by trivial name, e.g., hyaluronic acid. The former trivial names are also updated, e.g., chondroitin sulfate A changed to chondroitin 4-sulfate, etc. The complexes formed by the covalent linkage of the glycosaminoglycans to a polypeptide will be known as "proteoglycans" rather than polysaccharide-protein complexes. The glycan moiety of the macromolecules known by the name proteoglycans are well defined polysaccharide entities and, when specific attention is required in the discussion, the prefix "proteo" will be attached to the trivial name of the polysaccharide, e.g., proteokeratan sulfate (PKS), proteochondroitin 6-sulfate (PCh 6-S), etc. In case more than one type of glycosaminoglycan is covalently bound to a protein, the name will be, for example, proteokeratan chondroitin 4-sulfate (PKS-Ch 4-S). The glycoproteins will include those carbohydrate-protein complexes that fit Gottschalk's definition described in the text (page 33). There is no synonymous term for the carbohydrate component of a glycoprotein like glycosaminoglycan for the polysaccharide portion of a proteoglycan. Hence, with regard to the histochemical reactions with the carbohydrate components of both proteoglycans and glycoproteins, the term glycoprotein will be used with the understanding that in this instance reference is being made to its carbohydrate moiety. An additional term not found in Balazs' nomenclature will be "neutral heteroglycan." It is felt that as neutral mucopolysaccharides were distinct entities in older classifications, some term should be used in reference to those polysaccharides described as being composed of neutral hexoses and hexosamines.

The origin, location and identification of glycoproteins and glycosaminoglycan moities of the proteoglycans distributed within the

periodontal fibrous connective tissue has also been achieved with radioautographic methods (Mancini et al., 1961; Baumhammers and Stallard, 1968), histochemical methods (Dewar, 1955; Quintarelli, 1960; Engel et al., 1960; Fullmer, 1961) and biochemical methods (Schultz-Haudt et al., 1961, 1964, 1965; Thornard and Blustein, 1965; Kofoed and Bozzini, 1970; Munemoto et al., 1970). Similar to other types of connective tissue, glycoproteins and glycosaminoglycans of the gingival periodontal ligament are important structural components. However, much more is known about the glycosaminoglycans because with biochemical methods they are isolated from all types of connective tissue with greater ease than are the glycoproteins. Hence, the glycosaminoglycans isolated biochemically from the gingival periodontal ligament have been identified as Hyaluronic acid (HA), Chondroitin 4-sulfate (Ch 4-S), Chondroitin 6-sulfate (Ch 6-S), Dermatan sulfate (DS), Heparan sulfate (HS), and Heparin (H) (Table 2). All of these are polymers of a regular repeating disaccharide composed of a uronic acid and an amino sugar which, in most of these polysaccharides, has an attached sulfate group (Figure 5, page 49).

The neutral heteroglycans are considered as having a structure similar to the glycosaminoglycans except that the hexosamines never have an attached sulfate group and the hexuronic component is replaced by a neutral sugar (Zugibe, 1970).

Hexuronic acids are derived from simple hexose sugars by oxidation of $C_6$ to a carboxyl group. This group confers weakly acidic properties on polymers containing it, provided it is free to ionize. A hexosamine is derived from a hexose sugar by the replacement of one of the hydroxyl groups (usually that on $C_2$), by an amino group, $NH_2$ (White, Handler and Smith, 1968). In addition, the amino group usually has an acetyl group attached so that the resulting structure (-NH . CO . $CH_3$)

at the pH of tissue does not attach a proton and cannot become positively charged or cationic (Schubert, 1964). The hexosamines that are sulfated have this acid group attached by an ester link to either $C_4$ or $C_6$ of the pyranose ring structure (Hoffman et al., 1958a, 1958b; Jeanloz and Stoffyn, 1958; Suzuki, 1960). The acidic character of heparan sulfate and heparin is enhanced by replacement of the hexosamine acetyl group with a sulfate group (Brimacombe and Webber, 1964; Cifonelli, 1968). The regular distribution of these sulfate and/or carboxylate groups along the polysaccharide chains confers a common polyanionic character to all the glycosaminoglycans. These anions are always associated with an equivalent number of cations, called counterions, which can readily be exchanged with other tissue cations, depending upon their concentration and valence (Schubert, 1964). The counterion exchange is the basis upon which histochemical cationic dye molecules attach to anionic groups of the glycosaminoglycans, thus producing in situ staining. The more cations present as in trivalent dyes, the greater is the competition for the glycosaminoglycan's anions and the more anions present as with chondroitin and dermatan sulfates, the greater will be the intensity of staining. However, other tissue components with anionic groups would also stain. Therefore, agents with specific action against the glycosaminoglycans must be employed as controls in order to determine what area of the tissue section has stained as a result of the presence of glycosaminoglycans. A frequently used method is the prevention of glycosaminoglycan counterion binding by treatment of tissue sections with methanol containing HCl (Fisher and Lillie, 1954). This treatment is known as methylation and it completely removes sulfate groups by producing free acid methyl sulfates; but the carboxyl anions are only temporarily blocked by their on site conversion to methyl esters.

Biochemical studies of glycosaminoglycans isolated from different types of connective tissue (Meyer et al., 1956) and rat tissues in particular (Kofoed et al., 1968; Kofoed and Bozzini, 1970) have provided evidence suggesting that the proportion of each glycosaminoglycan varies with the histologic appearance of the tissue. The presence of about 20 percent of Ch 6-S in embryonic pig skin and its decrease to just a trace in the adult (Loewi and Meyer, 1958) suggests that Ch 6-S appears in conjunction with the fine immature collagen fibrils. In rat tissue, Ch 4-S was by far the major constituent in tracheal cartilage, which has proportionately more ground substance and cells than collagen fibers, whereas the connective tissues of rat skin and gingiva have more collagen fibers in proportion to ground substance and a high content of hyaluronic acid followed by a lesser amount of dermatan sulfate.

As mentioned previously, the glycosaminoglycans do not occur in tissues as free polysaccharides but exist in the native state linked to varying amounts of a non-collagenous protein. The sulfated glycosaminoglycans are covalently linked to the protein by way of a glycosidic bond between a terminal xylose residue and the hydroxyl of serine (Rodén and Armand, 1966). The structure of the linkage region is illustrated in Figure 3 (page 22). The non-sulfated hyaluronic acid may have either an electrostatic or covalent linkage to the associated protein. Several recent studies on animal HA tend to favour its covalent binding to the protein (Wardi et al., 1966; Margolis, 1967; Swann, 1968; Rodén and Mathews, 1968). However, until a linkage region has been isolated and characterized, a covalent link between hyaluronate and protein cannot be resolved (Laurent, 1970). These compounds of carbohydrate and protein known as proteoglycans would have definite shapes and functions in connective tissue.

From studies using various techniques, it has been deduced that in solution, proteohyaluronic acid and the proteochondroitin sulfates extend as diffuse molecules throughout a volume of the solution called their domain that is large in comparison to the weight of the molecule (Schubert, 1964). Data from sedimentation and streaming birefringence measurements (Ogston and Stanier, 1951, 1953) along with light scattering measurements (Laurent and Gergely, 1955; Rowen et al., 1956) has confirmed that the proteohyaluronate molecule in solution has a shape in the configuration of a flexible, randomly coiled, long chain polymer occupying a spherical domain with a hydrodynamic volume $10^3$ times larger than the space occupied by the dehydrated proteoglycan. Experimental evidence (Laurent, 1957) indicates that hyaluronate can bind with hydration energy only a small fraction of its weight as water. Therefore, the majority of the domain of the hydrated molecule contains water trapped within the random coil configuration in addition to limited sized organic ions (Mathews, 1967). The size of the domain is equal to the degree of flexibility of the randomly coiled polymer and would logically be governed by the length of the proteohyaluronate molecule, the ionization of the attached carboxylate groups and the ionic composition of the solvent. In a solution of low ionic strength, the net charge of the hyaluronate polymer would be anionic, thus creating greater stiffness of the random coil due to mutual repulsion of like charges. Conversely, a diffusion of cationic molecules into the domain would result in a reduction of the net anionic charge of the polymer due to the accumulation of these counterions in close proximity to the polyanionic chain. As a result of this counterion shielding, the repelling force of the anionic charges is decreased and the stiffness of the polymer coil is reduced (Schubert and Hamerman, 1968). The random coil of the hyaluronate

molecule also acts as a sieve by regulating according to size the diffusion of different molecules. Studies by Laurent and Pietruszkiewicz (1961) on the retarding effect of hyaluronate against a series of particles varying in size demonstrated that the retarding effect was greatest against particles over 400 Angstroms. A model demonstrating the spatial relationship of the proteohyaluronate molecule and the probable manner of regulation of the passage of other molecules through the ground substance has been proposed by Balazs (1961) for the vitreous body. The macromolecular structure is pictured as consisting of a three dimensional fibrous network made up of collagen fibrils surrounding the proteohyaluronate random coils and other ground substance components. However, it is doubtful that a similar model of proteohyaluronate and collagen fibril spatial arrangements could exist in the gingival periodontal ligament which, by comparison to the vitreous body, has thick organized bundles of collagen fibers and decreased interfibrillar and intercellular spaces.

Mathews and Lozaityte (1958), using light scattering, viscosity, and enzymatic techniques, concluded that the chondroitin sulfate-protein molecule of cartilage exists as a rod-like macromolecular unit of four million molecular weight and 3700 Å in length. From this data, a model for the proteochondroitin molecule was conceived (Mathews, 1965) in the form of a central protein core approximately 4000 Å in length, with 50 - 60 curved polysaccharide chains, each of 50,000 molecular weight and contour length 1000 Å, extending perpendicular in all directions, thus giving a three dimensional branched structure like a test tube brush. The area occupied by this macromolecular structure is also known as its domain. A model patterned somewhat after this concept is illustrated in Figure 3. The protein chain is extended and stiffened, and the

polysaccharide chains, although they also have a slight rigidity due to the mutual repulsion of anionic charges, still retain the property of flexibility which is responsible for changes of shape with varying concentrations of counterions (Schubert and Hamerman, 1968). This conformation of parts is what gives the proteoglycans their property of viscosity (Mathews, 1967). Various hyaluronidases, testicular, bacterial and leech will selectively reduce the viscosity of proteochondroitin sulfate and/or proteohyaluronate by degrading their polysaccharide chains but not those of dermatan and heparan sulfates, thus affording a measure of specificity (Meyer et al., 1956; Brimacombe and Webber, 1964). For general specificity, papain has been employed as it digests a large percentage of the protein core of the proteoglycans which allows removal of the glycosaminoglycan-protein fragments from the interfibrillar and intercellular spaces of connective tissue with water washes (Saunders and Silverman, 1967).

Although there is as yet no definite supportive evidence of the physiologic properties proteoglycans perform in connective tissues, several functions other than the regulation of diffusing molecules have been proposed, such as: resistance to compression (Fessler, 1960), ion binding and transfer (Schubert and Hamerman, 1968), water retention for maintenance of turgor pressure (Rogers, 1961), tropocollagen aggregation into fibrils (Lowther and Toole, 1968), cell growth and differentiation (Campani et al., 1959), orientation of fibrous architecture (Meyer, 1960) and stabilization of collagen fibers (Mathews, 1965; Jackson and Bentley, 1968). The Mathews model described in the preceding paragraph is assumed to be orientated between collagen fibrils with the proteoglycan protein core in parallel alignment (Figure 3). The stabilization role is achieved probably by electrostatic binding of the terminal ends of the extended

polysaccharide arms to the collagen fibrils. Jackson and Bentley (1968) have argued that the length of the polysaccharide chains (1000 Å) prohibits a close spatial approximation between the closely aligned collagen fibrils composing the fiber bundle. They therefore proposed a modification of the Mathews proteochondroitin sulfate-collagen interaction. Following an initial self-aggregation of tropocollagen molecules into a fibril of self-limiting size, which is held together by covalent cross-links, further aggregation of several fibrils is produced by interaction with a firmly bound glycoprotein. Consequently, a closer spatial arrangement of the fibrils could be achieved by the shorter heterosaccharide units of the glycoprotein. Then, at a higher order of magnitude, proteochondroitin sulfates and proteodermatan sulfate would provide stability to even larger fibrillar aggregates (Figure 4). Electron microscope studies of glycosaminoglycans in various connective tissues of different mammalian species (Revel, 1964; Luft, 1964, 1965; Curran et al., 1965; Serafini-Fracassini and Smith, 1966; Matukas et al., 1967; Smith et al., 1967; Highton et al., 1968; Meyers et al., 1969) have given supportive morphological evidence of filamentous structures as well as small spherical bodies within the interfibrillar areas and amorphous granular deposits in close association to collagen fibrils.

Although such theoretical model systems of molecular morphology may explain the directional orientation of collagen fibrils composing fiber bundles in some types of connective tissue, it is possible that other steric relationships may exist in connective tissue of different species as well as in different types of connective tissue of the same species. The content of collagen, the stage of development, the protein-polysaccharide ratio and the concentration of other constituents within the tissue may also influence different forms of proteoglycan-collagen

Figure 3. Model of the Chondroitin Sulfate-Protein Macromolecular Structure. (Patterned after the concept of Mathews, 1965.) To avoid disarray, less than one-half of the Ch-S side chains are shown. The model consists of a central protein core to which are attached approximately sixty polysaccharide chains of 50,000 MW and 1000 Å contour length extending outward in all directions to adjacent collagen fibrils. The linkage sequence is suggested by Rodén and Armand (1966). The attachment to the collagen fibril is assumed to be by electrostatic binding. The position of the polysaccharide terminal ends at major banding sites, although highly speculative for Ch-S, is suggested by the orientation of the bovine vitreous body (Smith and Serafini-Fracassini, 1967).

AGAL = N-acetylgalactosamine
GA = glucuronic acid

Figure 4. Proposed Model of Collagen Fiber Stabilization by Mucoprotein and Mucopolysaccharide (from Jackson and Bentley, 1968. In: Treatise on Collagen, 2a. Edited by B. S. Gould, London: Academic Press. Courtesy of the Authors, pp. 189-214). The tropocollagen molecule self-aggregate into a collagen microfibril held together by covalent cross-links. The model then suggests that several microfibrils are held together by a firmly bound glycoprotein. At yet a higher order of magnitude, proteodermatan sulfate and proteochondroitin sulfate are seen as providing stability to even larger fibrillar aggregates.

MPS = mucopolysaccharide

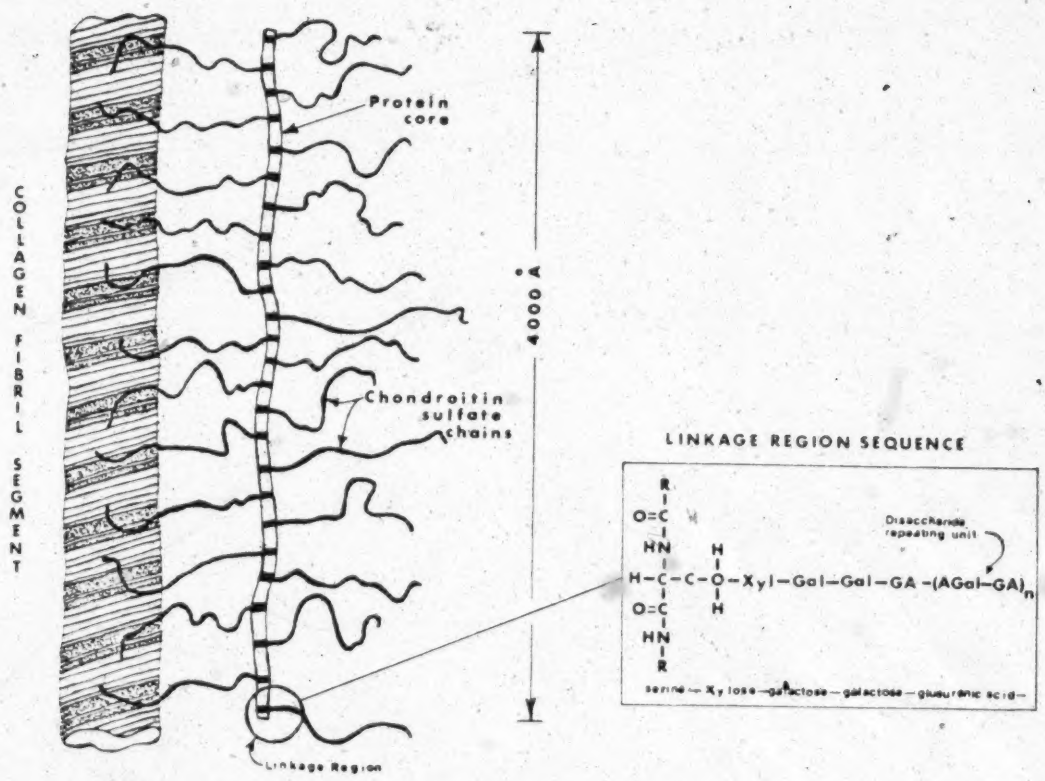



Figure 3

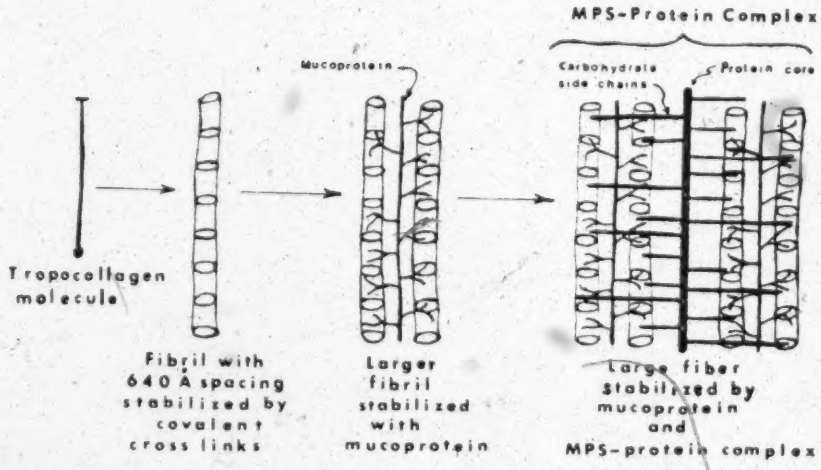

MPS-Protein Complex
Mucoprotein
Carbohydrate side chains
Protein core
Tropocollagen molecule
Fibril with 640 Å spacing stabilized by covalent cross links
Larger fibril stabilized with mucoprotein
Large fiber stabilized by mucoprotein and MPS-protein complex

Figure 4

spatial arrangements. In view of these assumptions, Schubert and Hamerman (1968) have proposed two diagrammatic proteoglycan models, one for bovine nasal cartilage and one for bovine nucleus pulposus. These models are similar to that proposed by Mathews (1965) with regard to a central protein core and attached polysaccharide side chains. However, they differ in that the protein core of the nucleus pulposus proteoglycan is about only one-third the length of the cartilage proteoglycan and both proteoglycans also have very short (200 Å) keratan sulfate chains attached to the protein core at periodic intervals between the longer (650 Å) chondroitin sulfate chains. In addition, other model systems have been proposed by Webber and Bayley (1956), Bernardi (1957), Fitton Jackson (1964) and Partridge (1966).

Albeit there have been light scattering, viscosity and enzymatic techniques to give some supportive evidence for the theory of the manner of alignment of a proteoglycan with collagen fibrils, thus suggesting a possible stabilizing role, a similar role for the glycoproteins must remain speculative at present due to lack of physical-chemical supportive evidence. Therefore, due to the scarcity of information, acceptance of any particular model as a standard for all connective tissue should be viewed with reservation.

Nevertheless, it is quite apparent that proteoglycans are an integral part of all types of connective tissue; and the appearance and function of different connective tissues depends not only upon the amount of fibrillar component but also upon the arrangement, the types and the proportions of the proteoglycans within the tissue. In addition, although much more supportive evidence is required, these preliminary studies previously referred to suggest that the proteoglycans have important structural and physiological roles in normal tissue, governed

mainly by the properties of the glycosaminoglycan moiety.

## The Problem

Due to these structural and functional roles, it could be suspected that the proteoglycans would undergo alteration in disease and perhaps are the first components of connective tissue to exhibit change. Even though gingival tissues present clinical normality, in many instances the connective tissue, when examined microscopically, exhibits a subclinical inflammatory response located adjacent to the boundary of crevicular and attachment epithelium. With retrograde change progressing to the observable disease state of clinical gingivitis, there is an apical migration of the epithelial attachment with concomitant destruction of the cementum inserted collagen fibers of the supra-alveolar region (Figure 2, fiber groups a, b and c). This destructive process, known as chronic gingivitis, proceeds slowly due to accompanying intermittent periods of tissue repair. What then would be the accompanying changes, if any, taking place in the proteoglycans? Are there quantitative changes of excessive amounts due to increased production by stimulation of fibroblasts as noted by the effects of early granulation tissue upon fibroblast cultures (Balazs and Holmgren, 1950); or are there decreased amounts of proteoglycans due to inhibition of formation? In addition, because of the intermittent reparative periods in chronic gingivitis, could there be significant qualitative changes similar to those noted in wound healing where the higher charged proteochondroitin sulfates and proteodermatan sulfate increase but the non-sulfated proteohyaluronic acid decreases during the early stages of healing (Bentley, 1968)? That is, with inflammation of an extended period, would there be a reversal of the normal ratios reported by

Kofoed and Bozzini (1970) of hyaluronic acid to dermatan sulfate in rat gingival fibrous connective tissue? If these alterations in proteoglycans of the gingival periodontal ligament do occur in disease, it is logical to assume that these changes should be observable with the electron microscope as changes in the macromolecular morphology providing, of course, that definite proteoglycan macromolecular structures peculiar to the gingival periodontal ligament do exist.

Therefore, the first prerequisite necessary for interpretation of deviations from normal is to establish in non-pathological gingival periodontal ligament the electron microscope appearance of different proteoglycans and the identity of their glycosaminoglycan moieties. One could assume that this information is already available by accepting the results of previous EM studies dealing with fibrous connective tissue (e.g., normal human knee synovial membrane; Highton et al., 1968; Myers et al., 1969) as being the same for the gingival periodontal ligament. However, species differences in the distribution of the chondroitin sulfates within the same site have been reported. Bovine aorta (Berenson, 1959) has significant proportions of Ch 4-S, but human aorta (Buddecke and Schubert, 1961) contains Ch 6-S as one of the major constituents. Likewise, pig skin has lower amounts of Ch 6-S than rat skin (Meyer et al., 1956). In addition, Munemoto et al. (1970) reported a combined content of Ch 4-S and Ch 6-S as being approximately twice as high as hyaluronic acid in isolates from bovine periodontal ligament, whereas calculations made from the results reported by Kofoed and Bozzini (1970) show that HA is around 60 percent higher than the chondroitin sulfates combined in rat gingival periodontal fibrous tissue. However, methods of isolation were different and the gingival samples also included labial mucosa connective tissue, which is of a looser

fibrous nature than the periodontal ligament fibers investigated by Munemoto et al. Nevertheless, it is apparent from the other studies cited that it is impossible to relate the types and proportions of glycosaminoglycans isolated from one species to a different species, even though the site of extraction has a relatively similar histologic appearance. Therefore, it is highly unlikely that the types and proportions of glycosaminoglycans would be similar for both synovial membrane and gingival periodontal ligament connective tissues; for not only are the connective tissues different histologically, but they are also located in different sites and different species.

It is not possible to conduct quantitative investigations with the electron microscope; but, theoretically, it should be possible to detect qualitative changes in the glycosaminoglycans if they are manifested as structural alterations. The first step would be to demonstrate glycosaminoglycan macromolecular structures in specimens of gingival periodontal ligament with the aid of a cationic electron dense compound. Of course, to prove ion-binding of the cation compound by the glycosaminoglycans, comparisons would have to be made with control specimens treated with an anionic blocking agent, followed by staining with the cationic dye. Then it should be possible to identify those structures composed of either hyaluronic acid or the chondroitin sulfates from those composed of dermatan sulfate and heparan sulfate by use of bacterial and testicular hyaluronidase followed by application of the electron dense compound. However, enzyme preparations are notorious for containing non-specific proteolytic contaminants; therefore, only those products specified as pure should be employed. An additional consideration which would influence the interpretation of enzymatic results is the presence of hybrid proteoglycan molecules. Formerly, it was thought that dermatan

sulfate was not the least bit affected by testicular hyaluronidase because it contains a different uronic acid than the other glycosaminoglycans. It is now established that disaccharide units of Ch 4-S and DS as well as Ch 6-S and DS exist in the same polysaccharide chains (Fransson and Rodén, 1967a, 1967b; Fransson, 1968). These authors also demonstrated that testicular hyaluronidase will, in fact, degrade a hybrid DS polysaccharide chain into products ranging from disaccharides to polysaccharide fragments of a molecular weight approaching that of the undegraded polymer due to glucuronic acid being an integral part of the molecule. There is also a strong possibility of Ch 4-S and Ch 6-S disaccharides being present in the same polysaccharide chain (Antonopoulos et al., 1965). However, as these biochemical investigations were on connective tissue from different sites than periodontal tissue and on different mammalian species, it is not known if similar conditions would exist in the gingival periodontal ligament. Assuming that glycosaminoglycan hybrids of chondroitin sulfates were present in the gingival periodontal ligament, it would not be possible to determine this in the electron microscope following hyaluronidase digestion for, as was noted earlier, testicular hyaluronidase activity distinguishes hyaluronic acid and both chondroitin sulfates from dermatan sulfate and heparan sulfate. That is, any particular glycosaminoglycan structure seen in sections with EM observation, which is then noted as being absent in control sections treated with testicular hyaluronidase, could be composed of any combination of either Ch 4-S, Ch 6-S and HA, or any one of these singly. It is unfortunate that at present there is no specific enzyme available for the histochemical differentiation between the two chondroitin sulfates.

Consideration must also be given to fibrous connective tissue structural glycoproteins (Robert et al., 1964), with their anionic

character as a result of the terminal position in the heterosaccharide chain of a sialic acid group (Gottschalk, 1966). These groups could be expected to produce complexes with solutions of cationic dyes into which tissue specimens have been immersed. Glycoproteins are considered to be firmly and closely associated with collagen fibrils (Bowes et al., 1957). Electron microscope studies (Myers et al., 1969) indicate that the glycosaminoglycan moities of the proteoglycans are also closely associated with collagen fibrils. Therefore, distinguishing between glycoproteins and proteoglycans, if both bound a cationic electron dense compound, would be difficult unless the enzyme neuraminidase was used prior to "staining" in order to degrade the sialic acid moities.

## The Purpose of the Investigation

Although there is a vast biochemical knowledge of the proteoglycans, the two prime considerations of function and shape in the native tissue are still somewhat speculative. So far, the EM demonstration of the shape of these structures has been with cationic electron dense compounds which are assumed to "stain" only the glycosaminoglycan moiety of the proteoglycan. This prevents the full visualization of the glycosaminoglycan-protein complex and leaves the models proposed by others somewhat unsubstantiated. Of greater concern is the inability to finitely identify the glycosaminoglycans as separate entities with enzyme histochemistry. However, the division of the glycosaminoglycans into groups of HA and ChS, DS, HS or HA, ChS and DS, HS with bacterial and testicular hyaluronidase, reduces the severity of this limitation.

Therefore, because of differences in the proportion of glycosaminoglycans in various connective tissues and their species differences in isolates from the same site, the purpose of this investigation will

be to determine the EM morphological nature and relative identity of glycosaminoglycans associated with gingival collagen fibrils of normal (that is, clinically free of disease) rat molar gingival periodontal ligament. As mentioned earlier, such a study of non-pathological tissue is an essential preliminary before any studies of diseased periodontal fibrous tissue are undertaken. Due to the similarity to the human molar periodontium, the rat molar was selected for study with the realization that significant findings regarding the structural identities of the glycosaminoglycans may not be similar to the human because of possible species differences. However, the identification of glycosaminoglycan structures in rat periodontal fibrous connective tissue would be a significant contribution towards the in situ location of the glycosaminoglycans already identified by biochemical techniques. To achieve this purpose, electron micrographs of sections from tissue treated with the highly cationic (+6) inorganic compound, ruthenium red, will be employed.

Ruthenium red, due to its cation charge, binds to tissue polyanions including the glycosaminoglycans but does not bind to sialic acid anions (Luft, 1971a), nor does it become electron dense unless combined with osmium tetroxide (Luft, 1964, 1965). A frequent criticism appearing in the literature is the poor penetration of ruthenium red into tissue specimens. To overcome this weakness in the method, perfusion of the animal with fixative containing ruthenium red will be included in this study. However, because of other tissue anions, any structures seen in sections from perfused tissues will have to be identified as glycosaminoglycans by comparison with sections from specimens treated with specific enzymes and the anionic blocking technique, methylation, before contact with the fixative and ruthenium red mixture.

Therefore, tissue specimens will have to be obtained surgically from the fresh state so that the enzymes and the HCl in methanol can be applied to unfixed tissues. To ensure that sections from these latter specimens have had sufficient penetration of ruthenium red, sections will be cut from only the outer 50 - 100 micron area of tissue blocks.

## Chapter 2

## RESUME OF THE LITERATURE

Although the main consideration in this study is the morphological nature of the gingival periodontal ligament glycosaminoglycans, they account for approximately only 5 percent of the total tissue dry weight (Meyer, 1955). Therefore, before reporting on the investigations which led to the present day knowledge of the identification and location of the glycosaminoglycans, consideration will be given to investigations of other ground substance components, such as the glycoproteins and neutral heteroglycans. These reports will include comparative studies on isolates from mucous secretions and from various connective tissues of diverse sites as well as studies of periodontal tissue glycoproteins and neutral heteroglycans. In order to familiarize the reader with the glycosaminoglycan group, certain features common to all will be given in addition to a detailed account of the individual chemical structures. As all types of connective tissue have been found to contain a spectrum of glycosaminoglycans in various amounts, and as the rat periodontal fibrous tissue contains all but keratan sulfate (Kofoed and Bozzini, 1970), the types and proportion of glycosaminoglycans in different connective tissue types will not be reported.

In addition, because the in situ identification of glycosaminoglycans has been achieved with the electron microscope using cationic dense compounds, a critique of these various methods will be included

in the discussion of the glycosaminoglycans. The evaluation of these methods will not only serve to introduce a comprehensive review of ruthenium red, but will also justify the use of Luft's ruthenium red method as the most favourable method for the demonstration of the macromolecular structures of gingival periodontal ligament glycosaminoglycans. The review of the historical use of ruthenium red for both light and electron microscope studies leading to the development of Luft's method and its popular usage will support this decision.

## Glycoproteins

Glycoproteins, according to Meyer (1938) (page 99) "contain hexosamine bound to protein or polypeptide in addition to other sugars. At present, the nature of the carbohydrate radical must be uncertain." Therefore, in this same report, according to the nature of the carbohydrate radical, he classified the glycoproteins as containing neutral mucopolysaccharides of unknown composition. Included in the group were the α and β ovomucoids, serum mucoid, serum glycoid and the globulins (egg white, serum thyroglobulins). At this time, only egg white and ovomucoid were known to contain glucosamine and mannose in the carbohydrate moiety. In 1945, Meyer reclassified these previous substances into two categories: mucoids or mucoproteins and glycoproteins. The mucoids or mucoproteins were defined as substances containing a mucopolysaccharide in firm chemical union with a peptide where the hexosamine content is greater than 4 percent. Into this group he placed the α and β ovomucoids, serum mucoid, serum glycoid and submaxillary mucoid which are now classed as glycoproteins. This has produced much confusion for, even up to the present day, mucoproteins and glycoproteins are terms used interchangeably to describe the same substances. The glycoprotein group in the 1945

classification included as examples serum albumin and serum globulins. He also redefined the glycoproteins as a group of proteins containing less than 4 percent hexosamine.

Since Meyer's classifications in 1938 and 1945, investigators have discovered six commonly found types of sugars occurring in the carbohydrate moiety of glycoproteins. These are D-mannose, D-galactose, L-fucose, D-neuraminic acid, D-glucosamine and D-galactosamine. The latter two are nearly always N-acetylated. In addition, it was discovered that different animal glycoproteins vary in (a) the number, compositon and size of their heterosaccharide groups, (b) the composition of the protein moiety, and (c) their carbohydrate content. Consequently, on the basis of common structural features, glycoproteins were redefined by Gottschalk (1966) as conjugated proteins containing as prosthetic group one or more heterosaccharides with a relatively low number of sugar residues (2 - 17), lacking a serially repeating unit bound covalently to the polypeptide chain. Hence, the overall building plan of a glycoprotein consists of a polypeptide core with heterosaccharides linked to functional groups of the side chains of aspartyl or glutamyl residues and probably also to the hydroxyl group of seryl (threonyl) residues by covalent bonds (for reviews, see Gottschalk, 1960, 1962, 1964, 1966).

Sialic acid is also an important component of most glycoproteins. The discovery of this acid in bovine submaxillary gland glycoprotein was made by Blix (1936); however, the acid was not named "sialic acid" until much later (Blix et al., 1952). During this interim period, as well as after, further investigations resulted in the identification of N-acetyl neuraminic acid, N-glycolylneuraminic acid and the diacetylneuraminic acids. These were found to be widely distributed in animal tissues and occur predominantly in a bound form (Blix et al., 1957). In this same

report, sialic acid was redefined as the group name for all these acylated neuraminic acids, neuraminic acid being the name of the parent unsubstituted compound. The presence of sialic acid and the absence of uronic acid is a striking feature. It is as if these two substances excluded each other in glycoproteins (Gottschalk, 1962). Structurally, the sialic acids can be considered as both a sugar and as an amino acid; and of all the component sugars of glycoproteins, it is the most characteristic one because it exerts a marked effect on the physical and chemical properties of glycoproteins. This effect is due to (a) the presence in the sialic acid molecule of a carboxyl group (pK = 2.6) which is always free and negatively charged at physiological pH, and (b) the location of the sialic acid group as the terminal non-reducing unit in the heterosaccharide chain remote from the protein moiety of the glycoprotein (Gottschalk, 1966). Therefore, if sialic acid molecules are sufficient in number, they impart acid properties to the whole glycoprotein complex (Melcher and Bowen, 1969).

Mucous Secretion Glycoproteins

In the protein moiety of the mucous secretion glycoproteins from bovine submaxillary gland (Hashimoto and Pigman, 1962), ovine submaxillary gland (Gottschalk and Simmonds, 1960), porcine submaxillary gland (Hashimoto, Hashimoto and Pigman, 1964), ovarian cyst fluid (Pusztai and Morgan, 1961), bovine cervix uteri (Gibbons, 1959), human cervix uteri (Gibbons and Roberts, 1963) and ovine intestine (Kent and Marsden, 1963), fifteen to sixteen amino acids have been identified and quantitated in varying amounts. While the amino acids threonine and serine, plus the pairs of proline and glutamic acid or proline and alanine, make up one-half or slightly more of the whole peptide chain,

minor quantitative differences occur in the amino acids glycine, valine, arginine, aspartic acid and leucine, which compose most of the remaining portion of the peptide chain. A more significant difference is in the percentage of carbohydrate from a low of 36 percent in ovine intestinal glycoprotein to a high of 90 percent in ovarian cyst fluid glycoprotein. In addition, these glycoproteins contain very little mannose and exhibit variations in the type and percentage amount of sialic acid, total hexosamine, glucosamine-galactosamine ratio and in amounts of galactose and fucose.

Connective Tissue Glycoproteins

Glycoproteins have also been extracted from all types of connective tissue. However, isolation in a satisfactory state of purity is a problem due to difficulty in separation from the proteoglycans and the need for enzymatic action to release some glycoproteins which are firmly associated with the fibrous proteins.

Eastoe and Eastoe (1954) characterized the protein moiety of ox compact bone glycoprotein as being composed of seventeen amino acids. They also determined the presence of the sugars mannose, xylose, glucosamine and galactosamine. Unfortunately, it is not possible to calculate from their results total hexosamines due to excess yields of galactosamine from chondroitin sulfate; nor is there mention of sialic acid determination in their report. The amino acids isolated in this study are quite different in content from the glycoprotein polypeptide of mucous secretions in that glutamic acid and aspartic acid rather than threonine and serine predominate.

Andrews and Herring (1965) were able to identify fifteen amino acids, N-acetyl neuraminic acid, N-glycolylneuraminic acid, galactose,

mannose, glucose, fucose, galactosamine and glucosamine composing a glycoprotein from bovine compact bone. Similar to the report of Eastoe and Eastoe, the amino acids of the protein moiety in the highest concentration were glutamic acid and aspartic acid. However, it is evident from a comparison of these two studies that individual quantitative differences occur between the amino acids leucine, valine, proline, lysine, glycine, threonine and serine which comprise almost all the remaining portion of the ox and bovine bone glycoprotein polypeptides.

The results of an investigation of bovine nasal cartilage glycoprotein by Partridge and Davis (1958) further demonstrate the quantitative variability of the polypeptide amino acids. Of the sixteen amino acids isolated from the protein moiety, greater amounts of glutamic acid and proline are found, while aspartic acid ranks fifth and threonine and serine are quite low on the list. In addition, the total hexosamine content is less than 2 percent. However, a later study by Rotstein et al. (1962) reports the presence of 10.4 percent reducing sugars and 1.2 percent sialic acid. Other studies, bovine aorta with a combined 11 percent hexose-hexosamine total and 7.1 percent sialic acid (Berenson and Fishkin, 1962), foetal calf skin with 9.7 percent hexose-hexosamine and 5.6 percent sialic acid (Bourrillon and Got, 1962) and spleen reticulin with 56.6 percent hexose-hexosamine but no sialic acid (Snellman, 1963), likewise demonstrate the variability of the carbohydrate moiety of glycoproteins extracted from diverse sites.

Evidence for the existence of a non-collagenous protein-polysaccharide complex and its firm association with the fibrous protein collagen is given by the study of Bowes et al. (1957). Two fractions containing both collagenous and non-collagenous protein were extracted

from the middle layer of young bovine skin with citrate buffer (pH 3.7) and dilute acetic acid (pH 2.8). The collagen protein was removed by NaCl precipitation and found to have a high hydroxyproline content and a low tyrosine content, which is typical of collagen protein. The remaining non-precipitated filtrates were analyzed for amino acid composition, hexosamine and hexose content. The proteins of the filtrates were assumed to be of non-collagenous types because they differed from typical collagen by having larger amounts of associated hexosamines, hexoses and high tyrosine values but low hydroxyproline content. In addition, the glycine, proline and arginine contents are much lower and the lysine, histidine, aspartic acid, leucine and valine contents are much higher than in collagen. The citrate soluble fraction was considered to be derived from the tissue fluids, blood vessels and muscle fibers; and it is possible that it represents small amounts of protein from a number of different sources rather than a specific protein. The acetic acid-soluble fraction contained larger amounts of glucosamine and hexoses and was assumed to be derived from a less soluble source. Indications of this non-collagenous protein's firm association to the collagen fibers is supported by the presence of some hydroxyproline which suggests that the collagen fibers were likewise very slowly going into solution. The acetic acid filtrate is also assumed to not be a glycosaminoglycan fraction on the basis that the latter is easily removed by alkali while at the same time a decrease in cohesion of the skin occurs. However, uronic acids were not detected in an alkali extracted non-collagenous protein included in this same study. Hence, its unequivocal identification as a glycosaminoglycan was not possible.

Although Bowes et al. do not term either of the acid soluble non-collagenous protein-polysaccharide complexes a glycoprotein, the

acetic acid soluble filtrate, on the basis of its firm association with collagen protein, could fit the description of connective tissue structural glycoproteins (SGP-S), as proposed by Robert et al. (1964). According to these workers, the SGP-S are contained in a residue obtained from connective tissue by first removing the soluble proteins with $CaCl_2$ and then collagen with trichloracetic acid (TCA). The TCA-insoluble portion contains the glycoproteins, a portion of which can be solubilized with urea. Thus, these glycoproteins are presumed to be tightly attached in the native tissue to the fibrous protein component and for this reason are called "structural glycoproteins."

Comparison of studies of SGP isolates from various tissues indicates that they vary in content (Robert and Robert, 1967), in quantity and ratio of sugar components, in percentages of sialic acid and in amino acid composition of the protein moiety (Moczar, Moczar and Robert, 1967; Moczar and Moczar, 1970; Robert and Robert, 1970). In one of these studies (Moczar and Moczar, 1970), a glycopeptide was separated from the insoluble structural glycoproteins of corneal stroma of several species and media of pig aorta in which the protein part does not contain hydroxyproline. This was assumed to indicate that heteropolysaccharide side chains exist in the insoluble fibrous network of connective tissues without being linked to collagen.

Periodontium Glycoproteins

Thornard and Blustein (1965) identified by colourmetric analysis sialic acid amounting to approximately 1.41 - 1.67 percent of the total mucopolysaccharide fraction in human gingival tissue. The tissue for this study was obtained surgically from patients suffering from chronic periodontitis and included both epithelial and fibrous connective

tissues. Therefore, the exact content of the sialic acid component associated solely with collagen periodontal fibers cannot be quantitated for it is also known that human epithelial cells grown in culture will elaborate sialic acid (Thornard et al., 1962). In addition, the possibility of contamination with serum glycoproteins containing sialic acid is a consideration in inflamed tissues. However, these investigators claim that the enzyme digestion employed in the study would liberate the sialic acid from the serum glycoprotein, thus rendering them dialysable and not present in the analysis.

Schultz-Haudt et al. (1965) report a slightly more thorough analysis of hydroxyproline containing glycopeptides from human gingiva, human aorta and skin from guinea pigs and rats. The analysis for sugar components of the glycopeptides indicated the presence of glucose, mannose and fucose in varying amounts in all isolates, whereas only some glycopeptides contained galactose, galactosamine and glucosamine. The report did not specify the types or amounts of these neutral and amino sugars contained in the individual glycopeptides. Hence, it is not possible to state the exact sugar composition of the gingival glycoproteins.

Sialic acid determinations were also carried out, but none could be demonstrated in any of the glycopeptides isolated. However, the ketosidic linkages joining the terminal sialic acid residues to the polysaccharide chains are sensitive even to 0.05 N mineral acid (Gottschalk, 1966). Therefore, their negative result with regard to sialic acid could be construed as either its non-existence in the native state or its loss from the tissues during their peptide separation procedures with a n-butanol-acetic acid-water mixture. With paper and gas chromatographic techniques, the amino acids, in addition to hydroxyproline and

proline, were identified as lysine, glycine, aspartic acid, glutamic acid, alanine, valine and leucine with smaller amounts of arginine, serine and tyrosine in all peptide isolates. Precise quantitations were not made, nor were sequential analyses undertaken. This is unfortunate, for it is not possible to make the same assumption as Bowes et al. (1957) that the peptides belong to non-collagenous proteins because they differ from collagen protein by having high tyrosine values but low hydroxyproline content. However, the presence of hydroxyproline in these glycopeptides supports the suggestion of Bowes et al. that its presence is probably due to collagen fibers slowly going into solution, thus indicating a firm association of the protein moiety of the glycopeptides with collagen fibers.

Although there are no physical chemical studies reported which deal with the interrelationship between collagen and glycoprotein, Bowes et al. (1955) suggest that during synthesis a glycoprotein might be incorporated into the collagen structure. The glycoprotein may even form an integral part of the collagen fiber (Bowes et al., 1956). The first step in the interaction would be a self-aggregation of tropocollagen molecules to a self-limiting microfibril size (Chapman et al., 1966; Vies and Bhatnagar, 1970). Further increase in fibril diameter is perhaps due to the combining action of glycoprotein. The fact that glycoproteins consist of short carbohydrate chains of 2 - 17 hexose units attached to the protein core which allows close spatial approximation, and the firm association of the glycoprotein to insoluble collagen fibers, does seem to give some support to this concept (Jackson and Bentley, 1968). The ultrastructural characteristics of the fibrils could also be regulated by the composition and amounts of glycoprotein. Robert and Robert (1967, 1970), using the examples of cornea with thin,

regular collagen fibers and sclera with thick, anastomosing and irregular fibers, theorize that because cornea is richer in a SGP with a variability in sugars compared to sclera, this is the reason for the differences in fibrillo-genesis.

## Neutral Heteroglycans

The neutral heteroglycans or heteropolysaccharides of skin derma, Achilles tendon, tracheal cartilage and bone matrix appear to have in common with glycoproteins a composition lacking in uronic acids. Also similar to glycoproteins, the polysaccharide chains contain the neutral sugars galactose, glucose, mannose and fucose, but differ in that there is a low content of hexosamine. These carbohydrate-containing materials stain with the periodic acid-Schiff (PAS) technique and thus are distinct from glycosaminoglycans (Glegg et al., 1954; Glegg, 1956). Windrum et al. (1955) reported the occurrence of galactose, mannose and glucose, but no hexuronic acid and only 0.16 - 0.17 percent hexosamine (glucosamine) associated with reticular tissue of human kidney. On the other hand, glycoproteins isolated from the various types of connective tissues are reported as containing 4.2 - 11.2 percent total hexosamine (Eastoe and Eastoe, 1954; Bowes et al., 1957; Banga and Balo, 1960; Bourrillon and Got, 1962; Snellman, 1963).

Leblond et al. (1957) investigated these fibrous connective tissue components stainable with the PAS method. They confirmed that the glycosaminoglycans are not reactive but rather it is a carbohydrate-protein complex that gives the reaction with the periodic acid-Schiff stain. These carbohydrate-protein complexes, when chemically analysed for sugar types, were found to be composed of galactose, fucose and mannose, as well as hexosamine and probably sialic acid. Each of these residues has

free 1 - 2 glycol groups capable of forming aldehyde groups by periodate oxidation which subsequently react with the Schiff's reagent to produce the colour.

Dische et al. (1958) isolated from bovine femur shaft ribs and ox vitreous body two types of neutral heteropolysaccharide-protein complexes free of uronic acid. One of these was soluble in alkaline ethanol and contained less hexosamine than the other insoluble heteropolysaccharide. The soluble fraction, although still combined with 40 percent protein, was further divided into two sub-fractions by dialysis. The dialysable portion was practically free of hexosamine and consisted of a mixture of galactose and glucose, with only a small amount of mannose and fucose. The non-dialysable part of the ethanol soluble carbohydrate contains, in addition to the three hexoses and perhaps small amounts of fucose, a significant amount of hexosamine, though less than the ethanol insoluble type of heteropolysaccharide. These workers suggest that the ethanol insoluble heteropolysaccharide is present in the ground substance combined with collagen fibers.

Schultz-Haudt et al. (1961) extracted from human gingiva two polysaccharide-protein complexes with different electrophoretic mobilities. These complexes were PAS positive, indicating the absence of glycosaminoglycans because under similar conditions controls of hyaluronic acid and chondroitin sulfate did not stain with PAS. Both fractions, in addition to glucosamine, contained the neutral sugars galactose, glucose, mannose, ribose and possibly fucose. These monosaccharides correspond largely with those of the "neutral heteropolysaccharides" of the connective tissue. However, glucuronic acid appeared to be a constituent of both complexes. The protein moiety of one of the complexes contained hydroxyproline, suggesting that the protein moiety was a soluble collagen and

the complex as a whole may represent some stage in the synthesis of collagenous fibers. Investigations of animal skin using the same techniques and following administration of radioactive proline also resulted in similar findings (Schultz-Haudt and Eeg-Larsen, 1961a, 1961b).

The results of similarly obtained extracts of carbohydrate-protein complexes from human gingiva are summarized by Schultz-Haudt (1965). Acetone-defatted tissue homogenates were subjected to the following polysaccharide protein extraction techniques: 5 percent aqueous trichloracetic acid, 0.14 M NaCl, 0.5 N - NaOH and trypsin, or acetate buffer-papain. The extracts were found to contain three substances which were separable by paper electrophoresis. The slowest migrating component stained with PAS and was therefore characterized as a glycoprotein. Only the protein of this complex in the TCA extract contained the amino acid hydroxyproline in amounts characteristic of collagen and reticulin. The other two substances, in order of electrophoretic mobility, were identified as hyaluronic acid-protein complex and chondroitin sulfate-protein complex. These were not associated with hydroxyproline but a non-collagenous protein of varying amounts, according to the method of extraction.

By careful evaluation of the previous literature cited, a critical analysis can be made of the neutral heteroglycans. For instance, methods used in some of the studies were not sensitive enough to quantitate hexosamine content as a basis for distinguishing from glycoproteins. Nor does the PAS-positive nature of neutral heteroglycans serve to distinguish histochemically between the glycoproteins for they too are PAS-positive due to a carbohydrate moiety composition of the neutral sugars mannose, galactose and fucose. In addition, the presence of glucuronic acid accompanying the neutral heteroglycans isolated by

Schultz-Haudt et al. (1961) would tend to indicate a contamination with hyaluronic acid. If quantitation of the glucuronic acid and glucosamine content proved to be in the approximate ratio of 1:1, this would support such a conclusion. This, however, was not done; hence, one can only speculate on the possibility of a contaminant. Furthermore, the absence of uronic acid does not prove that it was not present in the native state for this substance decomposes rapidly under conditions of acid hydrolysis (Windrum et al., 1955; Gardell, 1961). Likewise, a similar statement could be made for the absence of sialic acid as it too has a highly labile nature (Gottschalk, 1966). Therefore, the acceptance of a separate entity known as a neutral heteroglycan, being composed of a heteropolysaccharide-protein complex with a carbohydrate moiety composed solely of neutral sugars and a low hexosamine content, should be viewed with caution. It is perhaps possible for PAS stainable neutral polysaccharide chains linked loosely to a protein to be present in fibrous connective tissue ground substance as a result of the transient chemical population derived from the blood and local parenchymal cells. However, it seems more likely that, except for reticulin, neutral heteropolysaccharide-protein complexes native to connective tissue are, in fact, glycoproteins occurring either naturally without a sialic acid moiety or with a sialic acid component which has been lost due to methods of chemical isolation. The presence of hydroxyproline in the protein moiety of the gingival and skin extracts (Schultz-Haudt and Eeg-Larsen, 1961a, 1961b) probably represents collagen protein degradation as that found by Bowes et al. (1957) due to the methods necessary for extraction of the glycoproteins which are firmly bound to the collagen fibers.

## Glycosaminoglycans

The glycosaminoglycans of connective tissue listed in Table 2 (page 12) can be divided somewhat unequally into two groups according to the presence or absence of an ester linked sulfate group. The sulfated polysaccharides include the chondroitin sulfates, dermatan sulfate, keratan sulfate and heparan sulfate. An additional sulfated compound related to this group on the basis of its chemical similarity is heparin. However, as it is not a structural component in the same sense as these others found within the "amorphous ground substance," it will be given only brief mention in the discussion. The non-sulfated glycosaminoglycans are hyaluronic acid and chondroitin. In contrast to hyaluronic acid, chondroitin contains galactosamine and almost no sulfate (Davidson and Meyer, 1954) or approxomately 2 percent of ester sulfate (Meyer, 1960).

The structure of all the glycosaminoglycans is not as yet completely known. However, those whose structures are sufficiently known in detail seem to have the following features in common: (a) there is apparently no branching along the chain length; (b) the polysaccharides are polymers of a relatively high number of sugar residues, composed of a regular repeating unit of a disaccharide consisting of two different repeating units; (c) one of the monosaccharides is a hexosamine (glucosamine or galactosamine) and the other is either hexuronic acid (D-glucuronic or L-iduronic) or D-galactose; (d) the amino group of the hexosamine is never free but is usually acetylated and sometimes it is sulfated; (e) anionic groups (carboxylate or ester sulfate) are found regularly distributed along the polysaccharide length imparting a negative charge; (f) the anionic groups are usually associated with an equivalent number of cations or counterions consisting mainly of sodium ions under physiologic conditions. The polyanionic feature is

responsible for the glycosaminoglycans having the properties of polyelectrolytes (Schubert and Hamerman, 1968).

The present day knowledge of the structural components of the individual glycosaminoglycans as outlined in Table 3 is largely due to the efforts of Karl Meyer and his co-workers. The regular repeating disaccharides composed of the alternate monosaccharide units given in Table 3 are linearly linked by alternating $C_1 - C_3$ and $C_1 - C_4$ β-glycosidic bonds, except in the cases of heparin and heparan sulfate in which the linkages are α-glycosidic bonds (Mathews, 1967). These features, the chemical structure of the monosaccharides, the position of the anionic groups (carboxylate and sulfate) and the glycosidic bonds forming the disaccharides and tetrasaccharides of the glycosaminoglycans thus far isolated, are illustrated in Figure 5. The chemical structure of chondroitin has been omitted because so far it has only been isolated from the cornea and the structures of heparin and heparan sulfate are approximations based on reports by Brimacombe and Webber (1964) and Cifonelli (1968).

Hyaluronic Acid

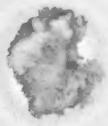
Hyaluronic acid is composed of equimolar parts of glucuronic acid and glucosamine (Meyer and Palmer, 1936). Its structure has been fairly well established as an unbranched polymer (Jeanloz and Forchielli, 1951) of the disaccharide repeating unit D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose (Weissmann and Meyer, 1954). The glucuronic acid moiety is located at the non-reducing end of the disaccharide (Weissmann et al., 1954). This would be the left side of the hyaluronate structure as shown in Figure 5. The glycosidic linkage of glucuronic acid to glucosamine is known as a β 1 - 3 glucuronidic linkage (Weissmann and Meyer, 1952). The

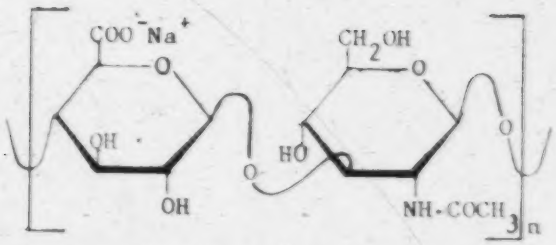
Table 3. The Structural Components of Connective Tissue Glycosaminoglycans (Data compiled from Meyer, 1955; Hoffman et al., 1958a; Rosen et al., 1960; Walker, 1961; Mathews, 1967).

| Glycosaminoglycan | Repeating unit | Position of Sulfate on Hexosamine | Glycosidic Linkages |
|---|---|---|---|
| Hyaluronic acid | D-glucuronic acid & N-acetylglucosamine | none present | GA-β(1-3)-AGlc[1]<br>AGlc-β(1-4)-GA[2] |
| Chondroitin | D-glucuronic acid & N-acetylgalactosamine | none present | GA-β(1-3)-AGal[1]<br>AGal-β(1-4)-GA[2] |
| Chondroitin 4-sulfate | D-glucuronic acid & N-acetylgalactosamine | C-4 | GA-β(1-3)-AGal[1]<br>AGal-β(1-4)-GA[2] |
| Chondroitin 6-sulfate | D-glucuronic acid & N-acetylgalactosamine | C-6 | |
| Dermatan sulfate | L-iduronic acid & N-acetylgalactosamine | C-4 | IdA-β(1-3)-AGal[1]<br>AGal-β(1-4)-IdA[2] |
| Keratan sulfate | D-galactose & N-acetylglucosamine also N-acetylgalactosamine | C-6 | Gal-β(1-4)-AGlc<br>AGlc-β(1-3)-Gal |
| Heparin | D-glucuronic acid N-sulfoglucosamine also L-iduronic acid | C-6, and N-sulfated; also 1/2 of uronic acids sulfated at C-? | SGlc-α(1-4)-GA<br>GA-α(1-4)-SGlc<br>SGlc-α(1-4)-SGA. |
| Heparan sulfate | D-glucuronic acid & N-acetylglucosamine | ½ N-sulfated | GA-α(1-4)-SGlc<br>SGlc-α(1-4)-GA<br>GA-α(1-4)-AGlc |

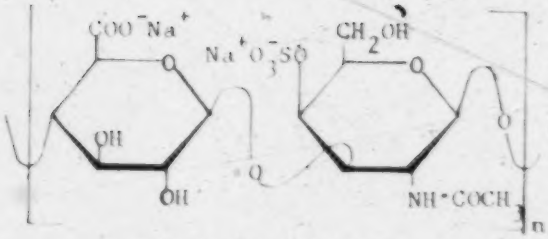
GA - glucuronic acid
AGlc - acetylglucosamine
AGal - acetylgalactosamine
IdA - iduronic acid
Gal - galactose
SGlc - N-sulfated glucosamine
SGA - sulfated glucuronic acid
[1] Hexuronidic linkage
[2] Hexosaminidic linkage

Figure 5. The Chemical Structures of the Connective Tissue Glycosaminoglycans Repeating Units (from Hoffman et al., 1958a; Hirano et al., 1961; Schubert, 1961; Schubert and Hamerman, 1968; Brimacombe and Webber, 1964; Citonelli, 1968).

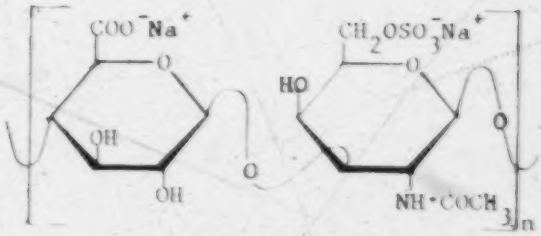




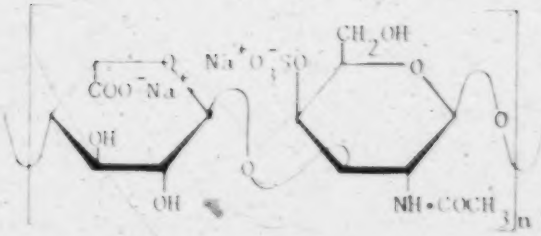
HYALURONIC ACID
n=about 2500



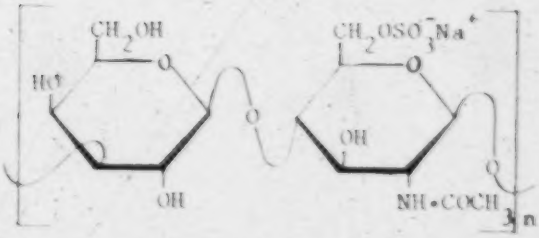
CHONDROITIN 4-SULFATE
n=about 60-65



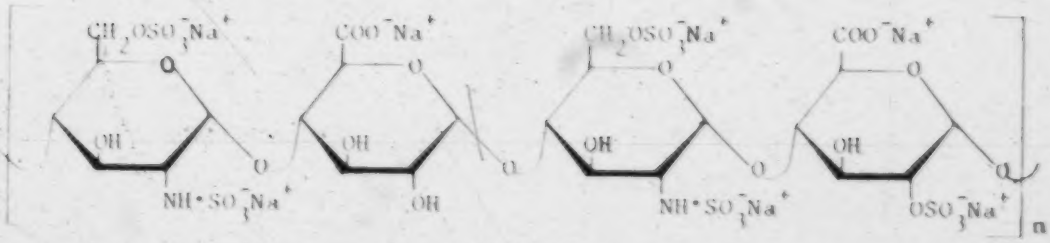
CHONDROITIN 6-SULFATE
n=similar to Ch 4-S



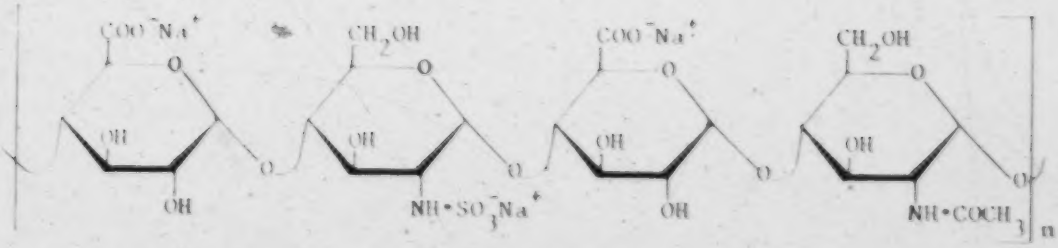
DERMATAN SULFATE
n=similar to Ch 4-S



KERATAN SULFATE
n=about 10 to 20



HEPARIN
n=about 10 to 15



HEPARAN SULFATE
n not known

polymerization of the individual disaccharide units is by glucosaminidic linkages of β configuration between $C_1$ of N-acetylglucosamine and $C_4$ of D-glucuronic acid (Meyer, 1955).

The Chondroitin Sulfates and Dermatan Sulfate

On the basis of solubility, optical rotation and enzymatic hydrolysis, three chondroitin sulfates are distinguished which were designated as A, B and C in the early literature (Meyer and Rapport, 1951). These are now termed chondroitin 4-sulfate (Ch 4-S), dermatan sulfate (DS) and chondroitin 6-sulfate (Ch 6-S) (Table 2, page 12). The fundamental repeating units of all three are composed of equimolar parts of uronic acid and N-acetylgalactosamine linked into disaccharides by a β 1 - 3 hexuronidic bond with the uronic acid moiety in the non-reducing position (Hoffman et al., 1958b). The disaccharides are polymerized to unbranched chains via β 1 - 4 hexosaminidic bonds (Hoffman et al., 1960). In Ch 4-S and DS, sulfation occurs in carbon 4 of the galactosamine (Hoffman et al., 1958b; Jeanloz and Stoffyn, 1958), in Ch 6-S on carbon 6 (Hoffman et al., 1958a; Suzuki, 1960). Although there are many similarities in the disaccharide repeating units of the two chondroitin sulfates and dermatan sulfate, there is a major difference in the character of the hexuronic acid moiety. In the chondroitin sulfates it is D-glucuronic acid (Meyer, 1955), but in dermatan sulfate it has been identified as L-iduronic acid (Jeanloz and Stoffyn, 1958). From the results of these studies and many others, the disaccharide units of Ch 4-S, Ch 6-S and DS can be formally described as:

β-1, 3-D-glucuronic acid + 2-acetamido-2-deoxy-4-O-sulfo-D-galactose

β-1, 3-D-glucuronic acid + 2-acetamido-2-deoxy-6-O-sulfo-D-galactose

β-1, 3-L-iduronic acid + 2-acetamido-2-deoxy-4-O-sulfo-D-galactose

However, such uniformity of glycosaminoglycan composition with repeating units of identical structure is open to question. For instance, the galactosamine moiety of the chondroitin sulfates may not always be sulfated at carbons 4 and 6, nor may they be the only positions of the sulfate groups. Preparations of Ch 6-S from shark cartilage have been reported with ratios of sulfate to hexosamine greater than unity, leading to the suggestion that sulfation also occurs on either $C_2$ or $C_3$ of the uronic acid moiety (Suzuki, 1960). Conversely, a Ch 4-S preparation from cornea had sulfate to hexosamine ratios greater than unity (Anseth and Laurent, 1961), indicating that all hexosamines are not sulfated. Unfortunately, current methods are inadequate for determination of the exact distribution of the disulfated and unsulfated disaccharides (Rodén, 1970). Hence, one could envision a polysaccharide with random disulfated or unsulfated hexosamines, or one with a preponderance of such residues in certain areas. However, rather than disulfation of repeating periods, most preparations from mammalian sources are somewhat deficient in sulfate which is thought to have little or no effect on the polyanionic properties of the polysaccharide (Rodén, 1970). In addition, there is a strong possibility that Ch 4-S and Ch 6-S disaccharides may be present in the same polysaccharide chain, thus ensuring a hybrid molecule (Antonopoulos et al., 1965).

Further heterogeneity manifests itself in the structure of the dermatan sulfate polysaccharide chains. Although iduronic acid was identified as the main uronic acid component of DS (Hoffman et al., 1956), glucuronic acid was also present in the preparation and remained in appreciable amounts in spite of extensive hyaluronidase treatment and repeated alcohol fractionation (Hoffman et al., 1957). The question then arose as to whether this fraction represents a contamination with a

different polysaccharide or a single molecular species of a hybrid nature with both L-iduronic and D-glucuronic acid residues. Fransson and Rodén (1967a) investigated this hybrid structure hypothesis for DS and their results indicate that glucuronic acid is an integral part of the DS molecule. Continued investigation (Fransson and Rodén, 1967b) of DS from pig skin provided evidence of a tetrasaccharide structure consisting of glucuronic acid + sulfated galactosamine -- iduronic acid + sulfated galactosamine with sulfate ester at $C_4$ of both galactosamine moities. A polysaccharide composed of tetrasaccharides with this structure could be considered as a co-polymer of Ch 4-S disaccharides and dermatan sulfate disaccharides.

Of further interest is a study by Fransson (1968) of a hybrid octasaccharide obtained from umbilical cord dermatan sulfate. This fraction contained glucuronic acid -- galactosamine disaccharides with the sultate at position $C_6$ of the hexosamine moiety and disaccharides of iduronic-galactosamine sulfated mainly at $C_4$ with occasional sulfation occurring also at $C_6$ of the hexosamine (that is, a polysaccharide containing Ch 6-S disaccharides and dermatan sulfate disaccharides sulfated at both $C_4$ and $C_6$ of N-acetyl galactosamine in the same chain).

Keratan Sulfate

Keratan sulfate (KS), regarding chain length, is probably the shortest connective tissue glycosaminoglycan. Like the other glycosaminoglycans discussed thus far, KS contains a repeating disaccharide unit, but an obvious difference is the absence of a uronic acid component. Instead, the first preparation of KS isolated from bovine cornea (Meyer et al., 1953) was found to contain equimolar proportions of D-galactose, N-acetyl glucosamine and sulfate. A second difference is the nature of

the glycosidic linkages between the monosaccharides forming the repeating period and between repeating periods forming the polymer, which are via β 1 - 4 and β 1 - 3 bonds respectively (Hirano et al., 1961). These latter workers were also able to place the sulfate ester at carbon 6 of the glucosamine moiety. However, sulfate has also been located at carbon 6 of the galactose moiety, thus giving preparations with slightly more than half of the total monosaccharides sulfated (Bhavanandan and Meyer, 1966, 1967, 1968). In addition, isolates of KS from cartilage and nucleus pulposus have been reported to contain galactosamine (Gregory and Rodén, 1961; Mathews and Cifonelli, 1965).

Another interesting feature of keratan sulfate is sialic acid up to 7 percent of some preparations (Schubert and Hamerman, 1968). The sialic acid is presumed to occupy terminal positions of side branches, but their exact location and distribution along the polysaccharide chain is still unknown (Rodén, 1970).

Thus, keratan sulfate has probably contributed to confusion in histochemical studies since it would show both metachromasia staining common to connective tissue glycosaminoglycans due to the anionic sulfate groups and PAS staining common to glycoproteins due to the neuttal sugar galactose and sialic acid (Schubert and Hamerman, 1968).

Heparan Sulfate

Heparan sulfate has tentatively been given the structure represented in Figure 5 (Linker and Sampson, 1960; Cifonelli, 1968). The tetrasaccharide structure shown is considered as a fragemnt expressing the characteristics of the polysaccharide and is not conceived as a strict uniform repeating unit. The composition of the unit was reported by Jorpes and Gardell (1948) as consisting of equimolar amounts of 2-

amino-2-deoxy-D-glucose, a hexuronic acid, acetyl and sulfate residues. Brown (1957) identified the uronic acid as D-glucuronic acid; however, residues of L-iduronic acid have also been reported (Cifonelli and Dorfman, 1962; Radhakreshnamurthy and Berenson, 1963; Lindahl, 1966). The monosaccharides are all linked by 1 - 4 glycosidic bonds (Muir, 1964) of the α configuration (Brimacombe and Webber, 1964).

The position of the sulfate group differs from the other sulfated glycosaminoglycans thus far discussed by being attached to $C_2$ of the hexosamine with the amino group forming a sulfamino group (Linker et al., 1958). They also concluded that approximately only one-half of the hexosamines possess the sulfamino group, the rest having the usual acetylamino group.

Disulfation of some hexosamines is also thought to occur by substitution of sulfate ester groups at both $C_2$ and $C_4$ or both $C_2$ and $C_6$ (Brimacombe and Webber, 1964). The exact distribution of the N-sulfated and N-acetylated units is not definitely established.

The results of studies by Cifonelli (1968) suggest that sections of the polysaccharide located near the linkage to the protein moiety have alternating N-acetylated and N-sulfated residues, but the majority of hexosamines distal to the protein are N-acetylated. Nor is it certain that branching of the molecule does not exist (Linker and Sampson, 1960).

It has been hypothesized that HS is a precursor of heparin (Cifonelli, 1970) as both are similar by having L-iduronic components, N-sulfated hexosamine units and α 1 - 4 glycosidic bonds. By comparison of the two tetrasaccharides illustrated in Figure 5 it can be assumed that the only difference is in the degree of sulfation. If this hypothesis is true, the source of heparan sulfate and heparin should be from the same cell. It is assumed that the site of synthesis or storage of

heparin is the mast cell because the glycosaminoglycans within the cell consist mainly of heparin (Schiller and Dorfman, 1959). However, very few mast cells are found in the intima and media of the aorta (Bertalsen and Jensen, 1960) which contains heparan sulfate but no trace of heparin (Kaplan and Meyer, 1960), thus indicating a different cell source for heparan sulfate.

Nevertheless, whether or not heparan sulfate is a precursor of heparin, much more investigation is required in order to elucidate the structure of both substances.

Molecular Weights

All of the glycosaminoglycans in Figure 5 have as their degree of polymerization an approximate number "n" of repeating units in the polysaccharide chain which is also an indication of their molecular weights. Therefore, a single molecule of hyaluronic acid with a molecular weight of $1 \times 10^6$ would consist of 2500 periods and have a chain length of 2.5 microns (Schubert and Hamerman, 1968). However, the molecular weight of hyaluronic acid varies from $7.7 \times 10^4$ to $14 \times 10^6$, depending upon its source, method of extraction and method of determination. For example, HA from vitreous humor has had recorded values ranging from $7.7 \times 10^4$ to $1.7 \times 10^6$ (Laurent et al., 1960), whereas from human synovial fluid the molecular weight varied from $1.2 \times 10^6$ to $8.4 \times 10^6$ (Fessler et al., 1954; Balazs et al., 1967). The molecular weights of Ch 4-S chains have been found to vary between 14,300 (Marler and Davidson, 1965) and 50,000 (Mathews, 1953). Similar variations in chain weights of dermatan sulfate have been reported from 17,000 (Fransson and Rodén, 1967a) to 48,000 (Jacobs and Muir, 1963). The latter figure is also given for heparan sulfate by Jacobs and Muir.

Keratan sulfate with polysaccharide molecular weight averaging 6,000 - 20,000 (Antonopoulos and Gardell, 1963) is perhaps the smallest of the connective tissue glycosaminoglycans. Schubert and Hamerman (1968) attribute a repeating unit number of ten to the smallest figure of the range.

These varying ranges of "chain weight" would suggest that a particular proteoglycan has different glycosaminoglycan chain lengths in the same tissue as well as different lengths for different tissues (Muir, 1965), thus further demonstrating the heterogeneity of all the glycosaminoglycans. It should be emphasized, however, that the preparation of a pure intact polysaccharide of very high degree of polymerization and in 100 percent yield is an extremely difficult problem; therefore, all molecular weight data should be viewed with caution (Laurent, 1970). Nor does the physiochemical data obtained for isolated products necessarily reflect the situation in the intact tissue. Several factors such as "mechanical depolymerization" due to use of high speed homogenizers and action of lysosomal enzymes during extraction procedures may tend to change the native molecules (Rodén, 1970).

Microscopic Histochemical Identification Methods

The histochemical techniques for the light microscope demonstration of both acidic and neutral carbohydrate components of ground substance and extraneous cell coats are quite numerous. However, the most commonly used methods are the metachromatic thiazine dyes toluidine blue and azure A (Pearce, 1968), the copper phthalocyanin dye alcian blue (Steedman, 1950), metal ion binding with colloidal iron (Hale, 1946) and the oxidation method with the periodic acid-Schiff reagent (McManus, 1946; Hotchkiss, 1947). Although a number of tissue components,

including nucleic acids, phospholipids and proteins in addition to sulfate and carboxylate containing compounds, are stained by these methods, it is possible to determine staining specific for glycosaminoglycans and glycoproteins. The procedures necessary for proving specificity of staining are careful regulation of pH and concentration of the reagents in solution, as well as incubation of control specimens with substrate specific enzymes and HCl in methanol for blocking sulfate and carboxylate groups.

Of these light microscopic methods, the colloidal iron, alcian blue and periodic acid-Schiff (PAS) have been used for the electron microscopic demonstration of glycosaminoglycans and glycoproteins. Except for alcian blue, modifications of the techniques as used for light microscopic investigations were necessary for application to tissue specimens obtained for electron microscope study.

A number of electron microscope investigations for determination of the nature of intercellular, interfibrillar and cell coat components (Curran and Clark, 1963; Curran et al., 1965; Ohkura, 1966; Matukas et al., 1967; Rambourg and Leblond, 1967) have used a modification of the Hale's colloidal iron method as suggested by Mowry (1958). In these studies, the effectiveness of the stain has been investigated either individually or in comparison to alcian blue and a modified PAS technique, the periodic acid-silver methenamine method. However, the reports are conflicting regarding whether staining of thin specimen blocks (50 microns) before epon embedding or direct staining of ultrathin sections after embedding is the best method of application to overcome the poor penetrating quality of colloidal iron. Although dense spherical particles 25 - 50 Å in diameter are visible with electron microscopic observation when penetration into specimens is favourable, a considerable proportion

of the density is non-specific. In addition, background staining occurs in connective tissues due to difficulty in differentiating out the stain with 12 percent acetic acid. However, Curran et al. (1965) demonstrated that methylation of tissues rendered negative all sites normally showing positive reaction to colloidal iron in the electron microscope, indicating that the reactive material carried acid residues such as carboxyl or sulfate groups. Matukas et al. (1967) also concluded colloidal iron was specific for glycosaminoglycans from observation of decreased iron deposition in cartilage matrix following incubation of tissue specimens in testicular hyaluronidase. They claimed that by regulation of the pH at 2, the ionization of weakly acidic radicals of non-specific components will be suppressed so that only sulfate or carboxylate groups will react with the positively charged iron particles. It is unlikely that any great interaction with carboxyl groups would occur at this low pH for glucuronic carboxyls have a pK of 3.1 - 3.3 (Mathews, 1959) and, therefore, at a pH of 2 are mainly undissociated and have limited interaction.

The colloidal iron method may be further criticized by the lack of definite morphological structures as noted in the published electron micrographs of these reports. The iron particles measuring 25 - 50 Å were distributed as dense coats along the outer wall, within cells as scattered granules, clumps of granules within cartilage matrix and as granules present in moderate concentration between collagen fibrils of intestinal submucosa tissue.

Colloidal thorium, another positively charged micelle similar to colloidal iron, was used by Revel (1964) because its larger particle size (50 - 80 Å) produces deposits of higher density than colloidal iron. In favour of the material is the morphologic patterns produced by it

within cartilage matrix. These consist of clumps of dense particles with a peripheral linear array of particles which suggest an arrangement of carbohydrate side chains of a proteoglycan. However, this material and the method of its use also has limitations. The granularity of the thorium precludes the resolution of very fine detail at high magnifications and the direct staining of ultrathin sections necessary for maximum penetration of the thorium is laborious and results in greater non-specific interaction of the material through displacement if sections are subjected to rough treatment. In addition, the embedding material is restricted to methacrylate as epon or araldite does not allow satisfactory penetration of colloidal thorium. This embedding material limitation is a disadvantage for methacrylate decomposes under electron bombardment causing a drawing together of tissue components as a result of surface tension forces created by melting of the methacrylate (Pease, 1964).

The staining of tissue specimen blocks with alcian blue for electron microscopic study of glycosaminoglycans (Tice and Barrnett, 1962; Ohkura, 1966) produces satisfactory contrast in the amorphous ground substance between connective tissue cells and collagen fibrils. The density consisted of fine granules 50 Å in diameter arranged in a linear pattern and as electron-opaque aggregates. However, the density was mainly located in the outer surfaces of the specimen blocks indicating that deep penetration of alcian blue is not one of its virtues. In addition, to avoid non-specific uptake of dye molecules by weakly acidic groups, the dye solution has to be used at a pH of 2.5 or lower for optimum interaction with sulfated glycosaminoglycans.

The periodic acid-silver method was claimed by Rambourg and Leblond (1967) to be fairly specific for detection of glycoprotein

composition of cell coats at the surface of mammalian cells. It was noted earlier that these cell coats also became electron dense with colloidal iron, a compound shown to be specific for carboxyl groups, as indicated by its non-reaction after methylation (Curran et al., 1965). However, it is not likely that the carboxyl groups belong to glycosaminoglycans but rather to sialic acid moieties of glycoproteins for iron deposition at the cell surface was not abolished by prior testicular hyaluronidase incubation of the tissue specimen. Of particular interest is the deposition of silver particles around collagen fibrils. Although silver is also deposited around collagen fibrils in the absence of prior periodic acid treatment, these deposits are of an increased size when the complete PA-silver procedure is employed. This non-specific uptake of silver without acid treatment occurs with other structures as well and it then becomes necessary to compare tissue specimens subjected only to silver methenamine with specimens stained after periodic acid treatment. This could be considered as a weakness in the method for it adds to the other standard procedures necessary for determining specificity of reagent interaction with glycosaminoglycans or glycoproteins. In addition, Rambourg and Leblond point out that the fixative agent could also add to non-specific staining by either creating more tissue reducing groups or direct reduction of the silver solution.

In the selection of a suitable electron dense compound for the demonstration of glycosaminoglycans contained within gingival periodontal ligament, it is reasonable that certain desirable criterion should be formulated. These are: (a) the compound should be a cationic reagent with a high charge density; (b) the reagent in solution should be stable and capable of optimal reaction close to the physiologic pH in order to maintain minimal tissue alteration; (c) the method of use should be

simple and applicable to 1/2 millimeter tissue specimen blocks; (d) the reagent should be capable of completely penetrating the specimen blocks for uniform distribution of density; (e) the density produced should consist of a minimum sized particle (10 - 20 Å) that allows at high magnification good resolution of fine detail, thus enabling visualization of distinct morphologic patterns within interfibrillar spaces; and (f) there should be a minimal distribution of density in non-specific structures which can be distinguished by incubation of control specimens in specific substrate enzymes and the methylation reagent. Although it is acknowledged that an electron dense compound may not satisfy all these requirements, thus necessitating compromise, the colloidal iron and thorium, the alcian blue and the PA-silver techniques, as well as possessing the limitations already discussed, lack too many of these qualities to deem them suitable for use in this gingival periodontal ligament study.

Critical evaluation of two other techniques using electron dense compounds, bismuth nitrate (Serafini-Fracassini and Smith, 1966; Smith, Peters and Serafini-Fracassini, 1967) and phosphotungstic acid (Pease and Bouteille, 1971), also leads to the conclusion that these compounds are unfeasible for a study of periodontal ligament glycosaminoglycans. The bismuth nitrate technique is claimed to give good preservation of protein-polysaccharide complexes in cartilage; however, there could be drastic results in periodontal fibrous tissue due to the twenty-four hour exposure to 0.5 percent bismuth nitrate in 0.1 nitric acid before fixation. Phosphotungstic acid, although it has been claimed to be a specific stain for polysaccharides by binding to numerous exposed hydroxyl groups, does not possess this specificity unless it is used at a very low pH of 0.8 (Pease and Bouteille, 1971). However, binding to

hydroxyl groups of polysaccharides could occur not only with proteoglycans and glycoproteins but also with polysaccharides in general. Hence, determination of specific staining of carboxyl and sulfate groups of glycosaminoglycans is not possible with methylation. In addition, as staining is by direct application to sections, a special embedding resin, hydroxypropyl methacrylate, is preferred as epoxy embedments may block the staining reaction. Therefore, because of these criticisms, it is not likely that either of these methods would be satisfactory if similarly applied to gingival periodontal ligament.

Although several methods have been investigated and found lacking, there is one method which comes very close to meeting all the criterion formulated previously. This is the ruthenium red method developed by Luft (1964). Ruthenium red can be used at physiologic pH on tissue specimen blocks, its method of use is uncomplicated, good contrast is produced in tissues without need of counter staining, the density has good resolution at high magnifications due to a 11.1 A molecular size, and it produces excellent electron dense morphologic patterns in intertibrillar spaces as demonstrated by Highton et al. (1968) and Meyers et al. (1969).

However, ruthenium red does have its limitations. It reacts with a variety of tissue polyanions and it has a rather shallow depth of penetration into tissue specimens. A light microscopic photomicrograph published by Highton et al. of a thick section from a specimen stained with ruthenium red gives evidence of penetration to a depth of 50 - 100 microns from the periphery. If the study area is limited to this outer zone, decreased density due to inadequate penetration would not be a serious matter except at the innermost boundary to which ruthenium red has penetrated.

## Historical Data and Application of Ruthenium Red

Ruthenium red, originally discovered by Joly (1892), is a highly cationic, inorganic compound usually prepared synthetically from ruthenium trichloride with chloride atoms serving as the counterions. It is not a pigment; but, when dissolved in water, it produces an intensely coloured red solution (Luft, 1971a). Fletcher et al. (1961) studied the chemistry of ruthenium red and, from their results, interpreted that this compound is a trinuclear ionic complex formed by oxygen bridged ruthenium atoms in a linear configuration of Ru-O-Ru-O-Ru, which is associated with fourteen ammonia molecules. They give a molecular weight of 858.5 and propose the structural formula as:

$$\left[(NH_3)_5\,Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5\right]^{6+}$$

In addition, Fletcher et al. identified a second compound, ruthenium brown resulting from the oxidation of the ruthenium red cation.

$$\left[(NH_3)_5\,Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5\right]^{7+}$$

This compound is as easily reduced back to ruthenium red as it is oxidized from ruthenium red, thus creating a repeating valence cycle between +3 and +4 of one of the ruthenium atoms in the red and brown ionic complexes.

Spectrophotometry studies by Luft (1971a) demonstrate that commercial preparations of ruthenium red also contain ruthenium brown and ruthenium violet as impurities.

Ruthenium red was first described by Mangin (1893) as an optical histologic stain for pectin substances (polyuronosaccharides), gums and mucilages of plants. From this time, except for reports by Eisen (1897) and Heidenhain (1913), very little interest was shown in the dye by histologists, and it appears that the field of botany was the main

employer of the dye, as indicated by the literature of the following four decades (Mehta, 1925; Carré and Horne, 1927; Bonner, 1936; Preston, 1952).

At the end of this period, there was a revival of interest in this organic compound as a stain for light microscopic studies of animal tissues and bacteria.

Ericsson (1953) stained deparaffinized sections of loose connective tissue of guinea pigs with an acidic solution of ruthenium red and noted an intense colour varying from bright red to brownish red in intercellular areas. Tests on purified samples demonstrated that hyaluronic acid stained brown red whereas chondroitin sulfate and heparin took on a bright red to a red violet colour.

Pappas (1954) noted that the cell membranes of amoebae stained red with ruthenium red; however, because of earlier reports of its doubtful specificity for pectic substances exclusively (Mehta, 1925; Bonnet, 1936), he draws no definite conclusions as to the chemical nature of the cell membrane constituent that reacts with the ruthenium red.

Breslau (1962), in his study of polysaccharides in microorganisms, reported that ruthenium red heavily stains the capsule of some hemolytic streptococci and some pneumococci, presumably due to capsular hyaluronic acid content. He also takes into consideration these earlier reports that questioned the specificity of the dye for pectic substances. However, he supports the cytochemical value of ruthenium red if it is used with satisfactory controls.

Fasske and Steins (1965) found that as a histological stain of tissues containing glycosaminoglycans, ruthenium red used at pH 1.0 was superior to alcian blue and astra blue methods.

Yamada (1970) used alcian blue (pH 1.0) followed by ruthenium

red (pH 2.5) to stain several sulfated glycosaminoglycan containing structures of rat and mouse tissues. He also tested the competence of this dual staining technique for the histochemical characterization of sulfated glycosaminoglycans in general by the interpretation of staining variability observed in methylated sections before and after saponification as well as enzyme digestion of sections with hyaluronidase and neuraminidase. He concluded that the dual staining reactions of most sulfated glycosaminoglycans following these control procedures implies that alcian blue and ruthenium red react rather selectively for sulfate and carboxyl groupings respectively.

Although there was an earlier report of apparent increased density of Neurospora cell wall (Shatkin and Tatum, 1959) at the electron microscope level following ruthenium red staining of thin methacrylate sections, the first published electron micrograph of cell wall density attributed to ruthenium red staining was of a marine diatome (Reimann, 1961). The density was later (Reimann et al., 1965) shown to be located in the mucilaginous coat of the cell wall rather than the cell wall itself.

Schnepf (1961) and Scholtyseck (1964) also photographically demonstrated density produced by the effects of ruthenium red on sections after embedding. The investigation of the former concerned the Golgi elements of plant tissues and the latter concerned localizing glycogen in a parasite.

Gustafson and Pihl (1967a) employed ruthenium red as a vital stain on suspensions of peritoneal mast cells. The electron density, although relatively intense, was irregularly dispersed from cell to cell in the heparin containing granules. This marked variation in granule electron density was surmounted by using ruthenium red on

ultrathin sections of peritoneal mast cells embedded in the hydrophilic embedding material glycol methacrylate (Gustafson and Pihl, 1967b). In addition, this same section staining technique was used by Pihl et al. (1968) to demonstrate glycosaminoglycans in sections of rabbit ear cartilage likewise embedded in glycol methacrylate. However, Martinez-Palomo (1970) failed to reveal a surface coat in a number of epithelial tissues when ruthenium red was similarly applied to glycol methacrylate sections.

The previous experiments indicate that the density produced by ruthenium red staining is of variable strength and distribution but is likely to be more intense if single cells are exposed to the dye either before embedding or after embedding with a water soluble resin. None of these experiments included the pre-embedding exposure of single cells or tissue blocks to ruthenium red mixed with osmium tetroxide. The idea of combining ruthenium red with osmium tetroxide originated with Luft (1964). In a study of mouse and frog microvilli of intestinal epithelial cells, small pieces of tissue were fixed for one hour in cracked ice with 2.5 percent glutaraldehyde or acrolein in 0.067 M cacodylate buffer (pH 7.0 - 7.5) containing 500 ppm ruthenium red. Following rinses in the cacodylate buffer, the tissues were post-fixed for three hours at room temperature with 1.0 - 1.67 percent osmium tetroxide in 0.067 M cacodylate buffer (pH 7.0 - 7.5) also containing 500 ppm ruthenium red. Dehydration was with increasing strengths of ethanol and embedding in an epoxy resin. This procedure resulted in an amplification of electron density in the filamentous or "fuzz" layer of the plasma membranes of the microvilli cells. The dense deposit was easily visible in the electron microscope by virtue of its own mass without need for further staining with heavy metals. In the same study, ruthenium red stained a

substance which appears to cover collagen fibrils of mouse diaphragm tendon. This substance also seemed to cement the tendon collagen fibrils to nearby striated muscle cells. The cementing substance, because of its notable density, revealed the collagen fibril periodicity as a negative image.

In a further study, Luft (1965) fixed control specimens of frog xiphoid cartilage in buffered glutaraldehyde containing ruthenium red. The resulting bright red tissue was then dehydrated and embedded in an epoxy resin without exposure to osmium tetroxide. The red dye was seen to be located in the matrix of the cartilage when 1 - 2 micron sections were studied in the light microscope. When adjacent thin sections (1000 A) were examined in the electron microscope, no extra density was seen in those same red areas observed in the light microscope and, therefore, must have contained bound ruthenium red. However, when additional cartilage tissue specimens were post fixed in osmium tetroxide containing ruthenium red, the matrix appeared brownish black in the light microscope instead of red. Adjacent thin sections of matrix examined in the electron microscope revealed, without additional staining, short threads 50 Å in thickness joining together dense spherical deposits 300 Å in diameter. Luft suggests that this meshwork of spheres and threads represents the protein polysaccharide macromolecules of cartilage intercellular matrix.

Subsequent to these initial reports by Luft (1964, 1965), many other workers (Kelly, 1966; Rondarett, 1967; Morgan, 1968; Behnke, 1968; Rightom et al., 1968; Myers et al., 1969; Brooks, 1969; Grontowski et al., 1969; Martinez-Palomo et al., 1969; Dimmock, 1970; Fowler, 1970; Price, 1970; Hashimoto and Lever, 1970; Hashimoto, 1971) and including Luft himself (1966a, 1966b), have since used this same fixative-dye combination for the electron microscope demonstration of structures composed

partially or entirely of acid polysaccharide-protein complexes.

Some of the previous articles make direct references to the ruthenium red method as described by Luft, whereas others report employing various concentrations of the dye with cold (4° C) osmium tetroxide. Nevertheless, it is apparent from their published micrographs that, irrespective of staining solution concentration, density is equally satisfactory without additional staining with heavy metals. However, in those experiments with animal tissues, a common criticism was the weak and erratic results noted in sections taken from the center of the tissue specimen block. This is presumably due to poor penetration of the ruthenium red.

Luft (1971a) advocates that using the ruthenium red-osmium tetroxide solution at room temperature produces an intensification of density compared with that achieved with the lower temperature (0° C). The rationale for adherence to procedure at room temperature is discussed later.

## Specificity of Ruthenium Red

Mangin (1893) was impressed with the singular ability of ruthenium red to stain pectin substances. However, Mehta (1925) reports that specificity is not completely exclusive to pectin substances for α-, β-, and λ-oxycelluloses, hemicelluloses, gums, galactans, free lignan, mannan and amylohemicellulose also stain with ruthenium red. Bonner (1936) supports the non-specificity for pectins and maintains that ruthenium red is specific for the sixth carbon atom carboxyl groups.

Luft (1971a) did a qualitative experiment to critically evaluate the specificity of ruthenium red. Specificity was indicated if a precipitate resulted from adding a solution of dilute cacodylate buffer

containing 500 - 1000 ppm ruthenium red to solutions of a variety of test substances. If no precipitation occurred, the mixture was illuminated to observe possible Tyndall scattering due to a finely dispersed precipitate. The results were grouped into four classes: (1) no reaction determined by lack of both precipitate and Tyndall scattering; (2) weak turbidity (no obvious precipitates but Tyndall scattering); (3) strong turbidity (distinct but dispersed precipitate); and (4) flocculent precipitate identified by a heavy coarse precipitate.

There were fifty-seven substances tested and, according to Luft, the results indicate that ruthenium red is generally unreactive with neutral polysaccharides (e.g., soluble starch, inulin), neutral polypeptides (e.g., poly-L-hydroxyproline, poly-L-tyrosine), various test proteins (e.g., albumin, fibrinogen), a component of the repeating structure of hyaluronic acid (N-acetyl-D-glucosamine), sialic acid and pure glycoprotein. Those substances giving weak turbidity have high charge density but low molecular weight (e.g., poly-L-glutamic acid) whereas those substances with low charge density but high molecular weight (e.g., hyaluronic acid, poly-D-glutamic acid, chondroitin sulfate) give strong turbidity. The substances giving the strongest reaction with flocculent precipitate are polyanions with high charge density (e.g., pectic acid, heparin). The common denominator among these substances is high polymerization and a charge density greater than that provided by a substance extensively substituted with alcoholic hydroxyl groups such as the highly polymerized inulin and polyvinyl alcohol. Those substances tested by Luft that satisfy this requirement are hyaluronic acid, chondroitin sulfate, thrombin, poly-D-glutamic acid, poly-L-aspartic acid, nasal mucus, pectic acid, heparin and polyacrylic acid. It is also significant to note that purified samples of hyaluronic acid precipitated

the ruthenium brown component of ruthenium red similar to that noted by Persson (1953).

It has been observed in electron micrographs that density occurs in intercellular lipid droplets of tissues exposed to ruthenium red. In addition to the previously described qualitative experiment, Luft (1971a) investigated the reaction of ruthenium red with the following lipids: crude egg lecithin, its separated main components, lecithin, lysolecithin and phosphatidyl ethanolamine, purified phosphatidyl ethanolamine, purified phosphatidyl inosital, phosphatidyl serine, and cardiolipin. He also included in this study the fatty acids lauric acid, myristic acid, palmitic acid and the triglyceride tripalmitin. Luft reports that the results of this experiment demonstrate a strong reaction with the more soluble fatty acids and with all the phospholipids except lecithin.

Luft concludes that caution should be used in the interpretation of ruthenium red staining for ruthenium red precipitates not only a large number of polyanions but also phospholipids and fatty acids. However, the likelihood of encountering most of these in tissue spaces is very low unless they are introduced deliberately. There is a possibility of accidental leakage of cytoplasmic materials from damaged cells, and this should be kept in mind in instances of unusual extracellular density. Nevertheless, the probability is high that the extracellular density is associated with glycosaminoglycans due to the vigorous reaction with ruthenium red given by chondroitin sulfate and hyaluronic acid. Furthermore, one should also provide supportive evidence by employing enzymatic specificity techniques with hyaluronidase, for example, as well as blocking agents such as methylation and cetylpyridinium chloride.

## Mode of Action

The previous experiments of Luft explain the selectivity of ruthenium red as a stain in optical histology or histochemistry. However, they do not explain the nature of the chemical reaction between ruthenium red, osmium tetroxide and the tissue that results in an amplification of density easily seen in the electron microscope. Although this chemical reaction is not fully understood at present, Luft (1971a) proposes two interesting hypotheses for the mechanism of this reaction.

The first hypothesis, termed the "catalytic model," is based on the cyclic oxidation of ruthenium red to ruthenium brown and the reduction of ruthenium brown back to ruthenium red. It begins with the electrostatic or ionic linking of ruthenium red to a substrate, the tissue glycosaminoglycans. The binding expresses the specificity of the reaction and provides the required closeness of fit for the postulated catalytic electron transfer system. The ruthenium red is then oxidized to ruthenium brown by the osmium tetroxide. The ruthenium brown in turn captures an electron from the glycosaminoglycan substrate and becomes reduced back to ruthenium red while at the same time the substrate is oxidized. In the presence of more osmium tetroxide, the cycle is repeated until oxidation of the glycosaminoglycan is completed with an equivalent reduction of the osmium tetroxide to lower insoluble oxidation products. The ruthenium red thus functions as a catalyst in that one molecule of ruthenium red is able to transfer many electrons from the substrate to the osmium tetroxide receptor; hence, several molecules of osmium tetroxide may become reduced to build up mass in the region of the bound ruthenium red.

The catalytic model may or may not be plausible, but it is known

that ruthenium red, like other elements of the platinum group, shows pronounced oxidative catalytic activity (Griffith, 1967). In addition, model experiments (Luft, 1971a) have shown that solutions of ruthenium red and osmium tetroxide in cacodylate buffer (pH 7.3) slowly turn brown as does tissue fixed in the reagent. This solution colour change is thought to be an oxidation of ruthenium red to ruthenium brown by the equivalent reduction of the oxidizing agent, osmium tetroxide. Therefore, the slow colour change in solution, plus the complex reaction suggested by the catalytic model hypothesis, may account for the increased intensity and distribution of density when the ruthenium red-osmium tetroxide phase is used at the longer than usual time (three hours) at room temperature.

The second hypothesis, termed the "self-propagating model" by Luft, is proposed as an alternate explanation because of the discovery that density in sections under the electron microscope was low when the tissue fixed in glutaraldehyde containing ruthenium red was followed by plain buffered osmium tetroxide, whereas the density is high when ruthenium red is also included in the osmium tetroxide. Surely a strictly catalytic reaction would function as well with a molecule of ruthenium red previously bound to tissue during exposure to the ruthenium red glutaraldehyde mixture as with any molecules of ruthenium red contributed by the ruthenium red-osmium tetroxide mixture. Therefore, according to the self-propagating model, the initial reaction is similar to the catalytic model in that the binding of a ruthenium red molecule to the glycosaminoglycan is followed by oxidation of ruthenium red to ruthenium brown by osmium tetroxide, but the oxidation of the polysaccharide substrate generates a new carboxyl from sugar ring carbon and oxygen atoms at the site of attack, which binds a second ruthenium

molecule. Hence, additional osmium tetroxide is required to oxidize this second ruthenium red molecule and, in doing so, the osmium is reduced to a form which produces electron density along the glycosaminoglycan chain. This procedure repeats itself by self-perpetuation and ceases only with the depletion of ruthenium red needed for the propagation. Therefore, the explanation for the low density observed in tissues where ruthenium red is included only at the glutaraldehyde phase and not the osmium tetroxide phase is insufficient ruthenium red molecules for binding to newly formed carboxyl groups. The more ruthenium red molecules bound to the glycosaminoglycan, the greater will be the amount of mass density resulting from the reduction of osmium tetroxide in its oxidizing reaction of ruthenium red.

As a third alternate model, one could speculate that the production of electron density by the interaction between osmium tetroxide and ruthenium red begins with the direct oxidation of the glycosaminoglycan by osmium tetroxide. In this scheme, two preliminary events would have to occur as a result of the oxidation of the glycosaminoglycan. Firstly, additional binding sites would be created through the formation of new carboxyl groups and, secondly, the osmium would be reduced to an intermediate osmate ion form with a +6 valence. Then, with the binding of the ruthenium red molecules to these numerous sites on the glycosaminoglycan chain, a third step would be oxidation of ruthenium red to ruthenium brown by the osmate ion which in turn is further reduced to osmium dioxide with a +4 valence. However, if osmium tetroxide was the direct oxidative agent of the glycosaminoglycan, one would expect to observe equal electron density in sections whether ruthenium red follows plain buffered osmium tetroxide or whether ruthenium red is used with osmium tetroxide. It is obvious that osmium

does not function at this level for tissues fixed in osmium tetroxide followed by exposure to ruthenium red gave negative density when observed in the electron microscope. The slowly darkening solutions of osmium tetroxide and ruthenium red gives supportive evidence that ruthenium red is being oxidized by the osmium and this is quite likely the same place in the procedure that osmium functions in tissues.

Yamada (1970), in his report on the dual staining of sulfated glycosaminoglycans with alcian blue and ruthenium red, suggests that the binding interaction of ruthenium red cations with glycosaminoglycan carboxyl anions is by formation of a salt linkage occurring according to the schematic chemical formulae illustrated in Figure 6. In the diagram, the top formula is the structural formula of ruthenium red showing the positions of the many ammonia groups attached to each of the three oxygen bridged ruthenium atoms. From examination of step 1, it is obvious that Yamada has given each ruthenium atom a +2 valence, thus allowing binding of two carboxyl groups per ruthenium atom by replacement of two of the ammonia ligands.

In Yamada's original diagram, he indicated that each $R\text{-}COO^-,H^+$ group appears as a separate entity. However, all these groups would have to be linked via their "R" letters in order to satisfy the polymer structure of the glycosaminoglycan. It is not logical to assume that one ruthenium red molecule could extend itself to randomly bind to six carboxyl groups belonging to different glycosaminoglycan chains. The more conceivable arrangement would be one where the ruthenium red molecule is aligned to fit along the flexible glycosaminoglycan chain. Therefore, the reproduction of Yamada's hypothesis as illustrated in Figure 6 has had the carboxyl groups joined via the letter $R^1$ to the letter $R^2$ in order to simulate the disaccharide repeating unit of a

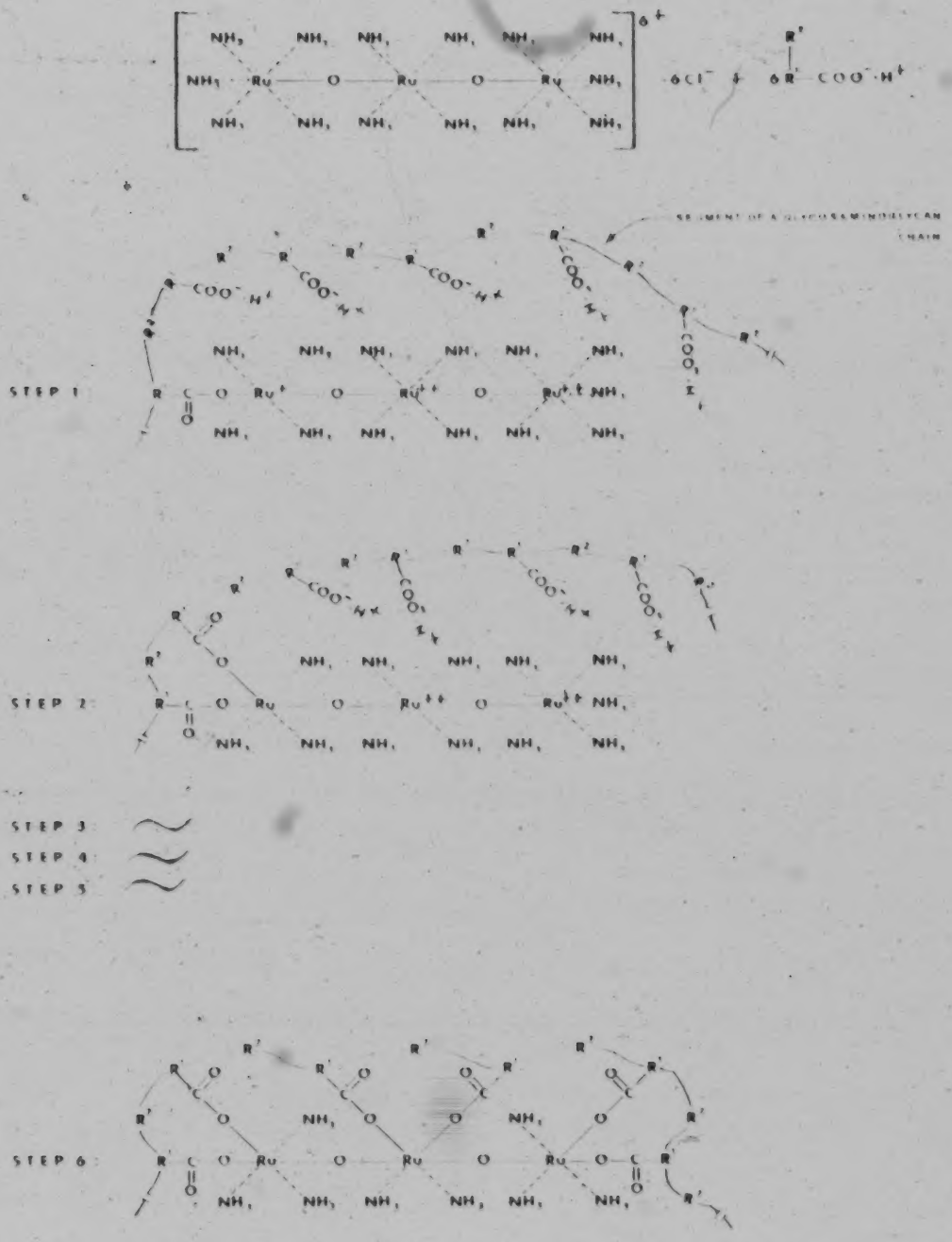



Figure 6. A Hypothesis for the Salt-linkage Formation Between Ruthenium Red Cations and the Glycosaminoglycan Carboxyl Anions. (Data from Yamada, 1970.) $R^1$ = hexuronic acid $R^2$ = hexosamine

glycosaminoglycan chain. The letter $R^1$ would represent the uronic acid component and the letter $R^2$ would represent the hexosamine component of the repeating unit.

In Yamada's technique, ruthenium red was used at pH 2.5 where he claims most carboxyls of glycosaminoglycans seem to be dissociated. His evidence for carboxyls being binding sites for ruthenium red molecules is the abolishing of ruthenium red staining by mild methylation and the return of staining following saponification of the methylated tissues. The loss of all negatively charged groups from a glycosaminoglycan by the methylation reaction and the return of negative charges to only the carboxylate groups by the action of saponification with dilute alkali is demonstrated by the two reactions given below (Schubert and Hametman, 1968).

Methylation

$$\begin{cases} O\text{-}SO_3^-\ Na^+ \\ COO^-\ Na^+ \end{cases} + 2CH_3OH + HCl \qquad \begin{cases} OH \\ COOCH_3 \end{cases} + CH_3OSO_3Na + NaCl + N_2O$$

Saponification

$$\begin{cases} OH \\ COOCH_3 \end{cases} + OH \qquad \begin{cases} OH \\ COO \end{cases} + CH_3OH$$

However, it was pointed out earlier in the discussion of colloidal iron that carboxyls of glucuronic acid have a pK of 3.1 – 3.3; hence, at a pH of 2.5, the majority of the carboxyl groups would be unassociated and therefore unreactive. The groups that would be mainly reactive at this pH are the sulfate groups of the galactosamines. The return of ruthenium red staining observed by Yamada after the mild methylation-saponification sequence is probably due to partially methanolyzed sulfate

esters (Spicer, 1960) that would add to the few carboxyl groups still dissociated to intensify staining. The probable explanation for mild methylation abolishing ruthenium red staining but still allowing a diminished alcian blue staining is that the tissue sections were first subjected to alcian blue, which binds to the partially methanolyzed sulfates, thus creating a situation where the ruthenium red would have to be strongly competitive in order to displace the bound alcian blue. Nevertheless, in spite of these conjectures, the maximum interaction between carboxyl anions and ruthenium red cations, as proposed by Yamada (Figure 6), would occur at a pH closer to neutrality than pH 2.5 due to the pK value of glucuronic carboxyls.

In addition, there is evidence of purified samples of hyaluronic acid reacting with ruthenium brown in vitro, whereas similar experiments showed that ruthenium red reacted with purified samples of chondroitin sulfate and heparin (Persson, 1951; Luft, 1971a). Furthermore, in acid solutions ruthenium red is easily oxidized to ruthenium brown (Fletcher et al., 1961). Therefore, the more optimal pH for ruthenium red binding to glycosaminoglycans for electron microscope studies would have to be either a very weakly acidic pH or, preferably, at neutrality in order to minimize oxidation to ruthenium brown. As mentioned previously, it is presumed from the slowly darkening solutions of ruthenium red and osmium tetroxide that electron density is a result of the oxidation of ruthenium red to ruthenium brown by osmium tetroxide which in turn becomes reduced to a lower insoluble form (Luft, 1971a). Hence, if this is the same reaction in tissues, ruthenium red, being the reduced form of the cationic molecule, would have to bind initially to the tissue anions rather than ruthenium brown as this latter compound is already oxidized and would not react with the oxidizing agent, osmium tetroxide.

However, it is not likely that ruthenium red binds to carboxyl groups as postulated by Yamada (1970). The carboxyl groups of both hyaluronic acid and chondroitin sulfate are 10 Å apart (Schubert and Hamerman, 1968); therefore, the binding of one ruthenium red molecule to six carboxyl groups would require the molecule to stretch over a distance of 50 Angstroms. Likewise, the binding of one ruthenium red molecule to both carboxylate and sulfate groups which are 5 Å apart in chondroitin sulfate (Schubert and Hamerman, 1968) would take up a distance of 25 Å for the six anions binding sites. As the molecular size of ruthenium red has been calculated at 11.3 Å (Luft, 1971a), the binding in both situations is a physical improbability.

A more plausible explanation for the binding of one ruthenium red molecule to anionic groups is suggested by Sterling (1970). Supported by crystal structure analysis, he reported that the staining group consists of a Ru ion coordinated octahedrally with four ammonia molecules in a square planer configuration and in the trans position to this plane are two $OH^-$ groups on either side of the Ru atom separated by a distance of 4.2 Angstroms. This staining group is located between the two oxygen bridges of the linear configuration of the ruthenium red molecule given by Fletcher et al. (1964). It was postulated by Sterling that specificity of staining is due to the host molecule having a staining site composed of two anionic groups 4.2 Å apart which will substitute themselves for the $OH^-$ groups.

Before any neat diagram can be drawn or any hypothesis accepted which attempts to explain the mechanism of the interaction between ruthenium red, the tissue glycosaminoglycans and osmium tetroxide, several questions would have to be provided with answers that are presently unknown.

Firstly, what is being oxidized to provide the electrons necessary to reduce ruthenium brown back to ruthenium red? According to Luft (1971a), the anions of sulfates are not the source of electrons for sulfates are fully oxidized already and carboxyls are highly resistant to oxidation. Therefore, a logical source would be the numerous exposed hydroxyl groups of the polysaccharide sugar rings.

A second question in need of an answer is: What are the effects of other tissue components upon the reduction reaction?

In addition, to what degree would fixation modify the extent of electron density produced by the overall process? Rambourg and Leblond (1967) noted that fixation in aldehyde increased the number of structures unspecifically stained with silver methenamine. This certainly indicates an unmasking of reactive sites. However, to what degree these unmasked sites could provide electrons for propagation of the reduction reaction once ruthenium red is bound and oxidized to ruthenium brown is also an unknown factor.

It is quite obvious, therefore, that many more tissue components will have to be biochemically tested with ruthenium red under a variety of conditions before the true nature of ruthenium red's mechanism of action is fully understood.

## Chapter 3

## MATERIALS AND METHODS

Specimens of gingival periodontal ligament from the mandibular molars of adult rats were chosen for the following reasons: (a) the diets of the rats can be controlled to maintain clinical health; (b) the molar dentition and periodontal supporting structures are similar to that of the human (Schour and Massler, 1949); (c) the supra-alveolar region is of an ideal miniature size for adequate penetration of fixatives for good morphological preservation at the electron microscope level; and (d) perfusion of fixatives into the vascular system of the animal can be performed with relative ease.

Glutaraldehyde was selected as the primary fixative and osmium tetroxide as the secondary fixative. Both fixative agents were diluted to the desired concentrations with sodium cacodylate buffer (pH 7.4). The cacodylate buffer was also used for rinsing tissue specimens between the primary and secondary fixation stages. All solutions were used clear or with the addition of ruthenium red (chloride salt, Gurr's) in the concentrations listed below:

1. 1.5 percent (v/v) glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) with 1500 ppm ruthenium red;
   3.0 percent (v/v) glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) with 3000 ppm ruthenium red.

2. 0.1 M Na cacodylate buffer (pH 7.4) with 800 ppm ruthenium red;
   0.1 M Na cacodylate buffer (pH 7.4) with 3000 ppm ruthenium red.

3. 2 percent (w/v) osmium tetroxide in 0.05 M Na cacodylate buffer (pH 7.4) with 400 ppm ruthenium red;

2 percent (w/v) osmium tetroxide in 0.05 M Na cacodylate buffer (pH 7.4) with 3000 ppm ruthenium red.

Specimens of gingival periodontal tissue as described diagrammatically by Figure 2 (page 5) were obtained from rats perfused with room temperature 1.2 percent glutaraldehyde in 0.1 M Na cacodylate buffer containing 1500 ppm ruthenium red. It was felt that this was a necessary preliminary in the initial trial phase of the experiment in order to overcome the uneven penetration of ruthenium red when conventional diffusion techniques are employed. Perfusion of the fixative-dye solution into the vascular system at a pressure between 100 - 120 millimeters on the physiograph gauge was accomplished via a canula inserted into the left ventricle of the heart. Within five minutes of continuous flow, the gingival tissue exhibited a pink hue from the ruthenium red; and after fifteen minutes of perfusion, the viscera were checked for the degree of fixation. This was deemed satisfactory and the flow of the perfusion fluid was terminated.

Upon completion of the perfusion technique, the mandibles were removed and approximately 1/2 millimeter thick specimens were obtained by discing in a bucco-lingual plane from the mesial of the first molar on through to the distal of the third molar. The discing was accomplished with a tooth and bone cutting machine (W. E. Niclas, Jamacia, N. Y.); and, as a coolant, a continuous flow of cold, fresh, perfusion fluid was run over the disc and the tissue being cut. The 1/2 millimeter specimens were then placed in a small jar containing 1.2 percent glutaraldehyde and 1500 ppm of ruthenium red mixture for an additional hour of fixation at 4° C. After this one hour period, the tissue slices were given three twenty minute rinses in cold (4° C) 0.1 M Na cacodylate buffer containing 800 ppm of ruthenium red. The specimens were then given a second period of fixation of four hours duration in cold (4° C) 2 percent osmium

tetroxide in 0.05 M Na cacodylate buffer containing 400 ppm of ruthenium red. Prior to the dehydration procedure, the specimens were briefly rinsed for five minutes in cold, clear 0.05 M Na cacodylate buffer. Dehydration was conducted over a period of three hours, commencing with cold 50 percent ethanol in water and finishing with room temperature absolute ethanol. Clearing of the alcohol was accomplished with three twenty minute rinses of propylene oxide and embedding of tissue specimens was in the epoxy resin, araldite. The cured specimen blocks were prepared for sectioning with a Porter-Blum ultra microtome (Sorvall model MT-2) by laboriously trimming away all hard tissues with razor blades, leaving the area of study contained within a trapezoid shape as shown in Figure 7. Sections approximately 600 - 900 Å in thickness were cut and the collagen fibrils and interfibrillar areas that were formerly in close proximity to the tooth surface were examined and photographed in a Philips 300 EM at an accelerating voltage of 80 kV using a double condensor illumination and a 50 micron objective aperature. Some sections were examined without further staining and others after staining with 5 percent aqueous uranyl acetate for thirty minutes, followed by Reynolds lead citrate for fifteen minutes.

For purposes of establishing a control, a second rat was also perfused in the same manner except ruthenium red was omitted from the perfusion fluid. The second step in the procedure, the cutting of tissue specimens, was also the same as for the other animal. In addition, the treatment of the 1/2 millimeter tissue sections with buffer rinse and osmium tetroxide was again similar to that previously described, except that ruthenium red was omitted from both solutions.

However, as specificity of glycosaminoglycans staining by ruthenium red must be established by the use of the methylation technique and

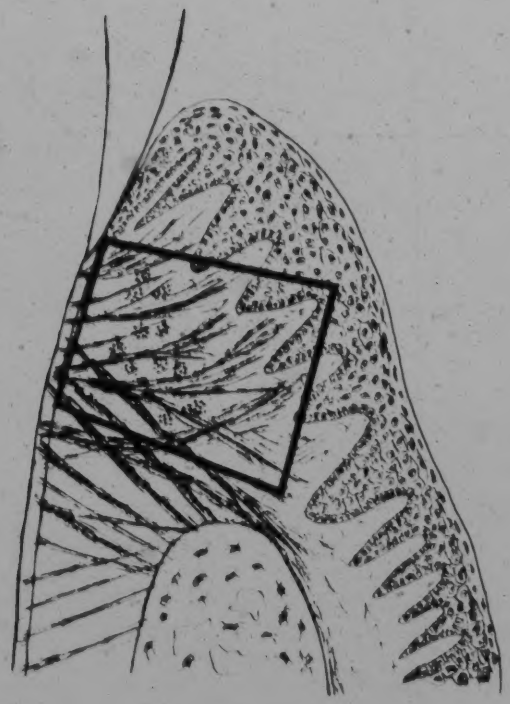


Figure 1. The Trapezoid Area of Study.

substrate specific enzymes acting upon unfixed tissues, a method of obtaining fresh gingival periodontal tissue had to be employed. The first method attempted in this investigation consisted of removal of the mandibles and cutting 1/2 millimeter sections with the bone and tooth cutting machine as described previously, with the exception that cold normal saline (pH 6.2) was used as a coolant. However, light microscopic and later electron microscopic studies showed this method to be unsatisfactory due to poor preservation of morphology. The probable reason for this problem is explained in the Appendix (page 145) where the method is written up in detail. Therefore, a second method was developed whereby tissue specimens were obtained by a gingivectomy technique. This procedure was also performed on excised mandibles in the fresh state. The removal of the tissue was accomplished by first making a circular horizontal incision around all three molars just below the level of the alveolar crest to a depth contacting the root. After making an incision interproximally, the tissue was reflected away from its attachment to the teeth by cutting into the cementum with a tiny spoon-shaped elevator. Once freed in this manner, the strip of supra-alveolar tissue was diced vertically into 1/2 - 1 millimeter sections for primary fixation with 3 percent glutaraldehyde in 0.1 M Na cacodylate buffer containing 5000 ppm of ruthenium red. It was felt that as the strength of both the glutaraldehyde and ruthenium red was increased from that formerly used (Appendix, page 145), a preliminary study not involving any controls should be undertaken in order to test the effectiveness of ruthenium red at 5000 ppm. However, after fixation of the tissue specimens in this solution for five hours, it was noted that the ruthenium red had been converted into tiny filamentous-like structures and the pH of the solution had dropped from 7.4 to 6.0. Nevertheless, it was decided that all the technical procedures

should be completed, including embedding, so that 1 micron sections could be studied with the light microscope for determination of the depth of ruthenium red penetration. The 1 micron sections from several specimens all showed poor penetration of ruthenium red. Hence, a solution of 3 percent glutaraldehyde in 0.1 M Na cacodylate buffer (7.4) was made up with a 3000 ppm of ruthenium red. After overnight storage at 4° C, the solution was checked for change in the pH and precipitate formation, neither of which had occurred. Therefore, fresh solutions of the glutaraldehyde fixative, cacodylate buffer rinse and osmium tetroxide fixative were prepared, each containing 3000 ppm of ruthenium red, for processing of additional gingival periodontal tissues. These tissue specimens were obtained by the formerly described gingivectomy technique from a rat of the same breed and dietary conditions as those previously sacrificed. The 1/2 - 1 millimeter sections were divided into five groups containing examples of buccal and lingual gingival periodontal tissue from all three molars of the right and left lower quadrants. The specimens were divided in this manner only for the sake of ensuring that each group would have sufficient numbers of evenly cut tissue blocks as both buccal and lingual gingival tissues appear the same histologically, regardless of the molar location. For identification purposes, the five groups were designated A, B, C, D and E. Individual specimen blocks belonging to each group were assigned an arabic number (A-1, A-2, etc.). The tissue specimens of each group were subjected to the following technical procedures formulated by Myers et al. (1969), except for slight modifications in the time of fixation and the concentration of ruthenium red:

Group A: Specimens of this group were fixed for five hours with cold (4° C) 3 percent glutaraldehyde in 0.1 M Na cacodylate buffer

(pH 7.4) containing 3000 ppm of ruthenium red, then post-fixed with 2 percent osmium tetroxide in 0.05 M Na cacodylate buffer (pH 7.4) containing 3000 ppm of ruthenium red for a period of three hours at 4° C. The excess osmium was cleared from the tissue with a brief rinse of cold 0.05 M Na cacodylate buffer. The specimens were dehydrated with increasing concentrations of cold ethyl alcohol in water, beginning with a strength of 50 percent and finishing with absolute ethanol which was brought to room temperature during the final ten minutes. Clearing of the ethyl alcohol was accomplished with three twenty minute rinses of propylene oxide and the tissue specimens were embedded in araldite.

Group B: This group of specimens served as the control and were fixed in the same concentrations of glutaraldehyde and osmium tetroxide as above but without the addition of ruthenium red.

Group C: Specimens of this group were placed directly into papain (Worthington Biochemicals Corporation, 130 units/ml, in normal saline) and incubated at 37° C for thirty minutes. The specimens were then briefly rinsed in room temperature 0.1 M Na cacodylate buffer (pH 7.4) before fixation and staining in the same manner as that described for the Group A specimens.

Group D: These specimens were incubated at 37° C in bovine testicular hyaluronidase (Worthington's, 1500 units/ml, in normal saline) for a period of one hour. Following incubation, the specimens were fixed and stained exactly as the Group A specimens.

Group E: This last group of the series was incubated at 37° C for one hour in normal saline and, like the papain and hyaluronidase fncubated specimens, was processed in the same manner as that outlined for the Group A tissue specimens.

With the completion of the curing of the araldite embedding

material, the specimen blocks were rough trimmed. Then, 1/2 micron sections were cut for study with the light microscope. The sections were first examined for penetration of ruthenium red which was satisfactory, and then re-examined after toluidine blue staining for the presence of cervicular epithelial cells and fragments of cementum along the former proximal border to the tooth. The specimen blocks demonstrating these features in their stained 1/2 micron sections were considered acceptable for further study with the electron microscope while all others were discarded.

Those specimen blocks from each group which were found suitable were further reduced to a manageable size for subsequent sectioning with the ultra microtome. Sections ranging in thickness from below 600 Å and above to 900 Å were cut and examined in the Philips 300 EM without further staining, except for the sections from specimen blocks processed without ruthenium red. Sections from these latter tissue specimens were collected on copper grids and stained with the following reagents:

1. A saturated solution of uranyl acetate in 50 percent methanol for thirty minutes.
2. Five percent aqueous uranyl acetate for thirty minutes, followed by Reynolds lead citrate for fifteen minutes.
3. Two percent aqueous phosphotungstic acid (pH 4.5) for thirty minutes.
4. Two percent aqueous phosphotungstic acid (pH 7.0) for thirty minutes.

As the main interest of study in all the specimens was of the periodontal collagen fibrils and interfibrillar spaces in close proximity to the coronal portion of the cementum and the apical portion of the epithelial attachment, the majority of micrographs were taken of areas as close as possible to the edge of the sections which would have bordered the tooth.

Chapter 4

# RESULTS

Thin sections of molar gingival periodontal ligament obtained from rats fixed by perfusion with glutaraldehyde and ruthenium red, followed by immersion of 1/2 millimeter tissue blocks in osmium tetroxide and ruthenium red, did not exhibit an intense electron density in association with collagen fibrils. Plate 1 is an example of an area of periodontal ligament collagen obtained from close to the middle of the specimen block which was prepared by this method. The most prominent electron density seen in this micrograph is in the form of circular-shaped deposits randomly distributed along the lateral border of a longitudinally sectioned collagen fibril. These dense deposits average 180 Å in length and 150 Å in width and are termed spherical masses (SM). A further manifestation due to the weak density is the indistinct periodicity of the collagen fibril which would appear as a pronounced negative image if heavier surface deposits of electron dense material were present surrounding the collagen fibril. All thin sections cut from several tissue blocks prepared initially by perfusion when examined did not exhibit any greater definable electron density in any area of the sections than that shown in Plate 1. In many instances it was difficult to determine the presence of distinct collagen fibrils. This necessitated counter staining of the sections with uranyl acetate and Reynolds lead citrate. Plate 2 shows a section stained with these heavy metals. Although it became much easier to study the collagen fibrils by

electron microscopy, the laterally located spherical masses were obscured by the counter stain. Nevertheless, the micrograph does demonstrate two filamentous structures composed of electron dense material extending at right angles from the collagen fibrils. These are termed fine filaments (FF) and measure approximately 50 Å in diameter.

The micrographs demonstrating the most remarkable electron dense structures in an area close to the cementum surface were taken of sections cut from tissue specimens obtained by gingivectomy which were fixed and stained by the conventional diffusion technique in the two ruthenium red containing solutions. The first of these, Plate 3, shows fine filaments (FF) measuring 30 - 45 Å in thickness which extend laterally from collagen fibrils surrounded by dense coats (DC). The collagen fibrils (C) appear as negative images lying within the mass of electron dense coats. Unfortunately, the margins of the collagen fibrils are not easily discernable and therefore it is not possible in this micrograph to determine the thicknesses of the dense coats. However, study of the fine filaments with a 10 power eye piece containing a scale calibrated in tenths of a millimeter indicate that these filaments are composed of linear arranged electron dense granules measuring approximately 13.6 Å in diameter. This corresponds very closely to the molecular size of 11.3 Å given for ruthenium red (Luft, 1971a). In addition, the fine filaments exhibit anastomosing (a) in some areas with periodic amorphous masses (AM) of electron density at the point of union of filaments, thus suggesting a network-like arrangement. The sharp density seen in this micrograph was made possible by the use of Kodalith, an emulsion which produces an extremely high contrast. As this type of film was in limited supply and difficult to obtain, stocks were soon exhausted and, therefore, it was not available for the making of other

micrographs included in the series.

As a result, the remainder of the plates are a grayish tone and not of the same degree of contrast due to lack of a full range of blacks and whites. Nevertheless, these micrographs do show collagen fibrils with distinct light and dark minor bands which collectively give the characteristic 640 Å periodicity. Fine filaments are also noted (FF arrows) in Plates 4, 5 and 7. These are of a larger diameter (50 Å) than those seen in Plate 3. The filaments extend between collagen fibrils and appear to link the fibrils together by attachment at a specific locus in the major band period. A net-like arrangement of the fine filaments is also evident at the extreme right middle of Plate 5. Of particular interest is Plate 4 where there appears to be a branching effect of one of the filamentous structures. The unbranched portion of the filament measures 110 Å which is approximately twice as wide as either of the two filaments resulting from the branching. Spherical masses first seen and described in Plate 1 are likewise noted in Plates 4, 5 and 7. However, the spherical masses seen in these latter plates, when several from each plate are averaged, are larger than those of Plate 1 by having a length extending along the collagen fibril of 200 Å and a width of 190 Angstroms. Where resolution permits, the spherical masses appear to be located at the darker portion of the major band repeating period of the collagen fibril. This is demonstrated in Plate 5 where one of these spherical masses is shown on the lateral side of a collagen fibril with one of the major bands marked off with broken lines. In addition, the right side of Plate 5 suggests that there is a relationship between fine filaments and spherical masses. Although sharp detail is difficult to define, due to low contrast and the thin diameter of the electron dense filaments, it appears that fine filaments are attached to

collagen fibrils via a union (u) with spherical masses. This same feature is likewise noted in Plate 4 (arrowhead). Also noted in Plates 4, 5, 6, 7 and 8 are dense coats (DC) seen as accumulations of electron dense granules closely associated with the collagen fibrils. The micrographs showing longitudinally sectioned collagen fibrils exhibit the dense coats in varying degrees of thickness, ranging from 100 - 200 Angstroms. The dense coats are most evident in Plate 6 where they are noted as electron dense envelopes surrounding the transverse sectioned collagen fibrils. In these areas of closely packed collagen fibrils, the dense coats appear to serve as an embedding media and thus link the collagen fibrils together. This linking arrangement is not haphazard as indicated by Plate 8 where the collagen fibrils in longitudinal section with interconnecting dense coats are orientated with their major banding sites in register.

When tissue specimens are fixed in glutaraldehyde and osmium tetroxide without the addition of ruthenium red, counter staining of thin sections is necessary to impart density and render the collagen fibrils visible in the electron microscope. Plate 9 is an example of a section stained with 2 percent phosphotungstic acid (pH 7.0) obtained from one of the tissue specimens prepared without ruthenium red. The minor banding of the collagen fibril is much more distinct as are the margins of the longitudinally sectioned fibrils than that noted in the previous micrographs. There is no evidence of dense coats, spherical masses, or fine filaments composed of electron dense material. The interfibrillar areas, however, do exhibit deposits of very fine density without specific structure and form. Sections counter stained with 2 percent PTA buffered to a pH of 4.5 were also examined and photographed. However, these sections, when viewed in the electron microscope, were

of extremely poor contrast and the micrographs made gave very little information due to the faint structures seen within. Similar observations of low contrast were noted in micrographs of sections stained in alcoholic uranyl acetate and other sections stained with aqueous uranyl acetate and Reynolds lead citrate, although they exhibited adequate contrast, were too contaminated with superimposed lead stained dust particles to be of any value. Hence, these latter micrographs of the three alternate staining methods were not included as part of the results.

Micrographs of sections from specimens incubated in bovine testicular hyaluronidase before staining in ruthenium red (Plates 10 and 11), when compared with the micrographs of sections from ruthenium red stained tissue specimens without prior incubation in hyaluronidase, exhibit a decrease in the amounts of the electron dense structures. The filamentous structures in particular are much fewer in number, nor is there any evidence of the net-like arrangement of fine filaments as seen in Plates 3 and 5. These filaments that are still evident in Plate 10 are of much greater diameter (85 - 105 Å) and therefore have been termed coarse filaments (CF). Of interest is that the coarse filament so labelled in Plate 10 appears to branch similar to the fine filament seen in Plate 4. The spherical masses, although still readily apparent in Plate 10, do appear to be fewer in number and slightly smaller in length (160 Å) and width (110 Å) than the average 200 Å length and 190 Å width of the spherical masses seen in Plates 4, 5 and 7. However, this diminution of size is difficult to equate as neither Plate 10 nor 11 contains sufficient numbers of collagen fibrils in longitudinal section to make a valid comparison. The dense coats surrounding the collagen fibrils are also quite evident in both Plates

10 and 11, but are best seen in Plate 11 surrounding collagen fibrils cut in transverse section (arrowheads). Measurements of the dense coats seen in Plate 11 indicates an average thickness of 82 Å which is less than the average thickness of 132 Å of the dense costs surrounding the transverse sectioned fibrils of Plate 6. Plate 11 also exhibits two fine filaments (arrows); however, it is quite evident that neither Plate 10 nor 11 demonstrates these fine filaments in the same quantities as seen in micrographs of sections not incubated in testicular hyaluronidase before staining with ruthenium red.

The area of the outer edge of the trapezoid-shaped section selected for study in all sections from specimens routinely stained with ruthenium red had to be carefully examined for collagen fibrils, which were not closely packed, in order to obtain micrographs demonstrating any interfibrillar electron dense structures. However, tissues incubated in papain before fixation and staining did not present this problem in identical areas. The majority of these sections exhibited easily defined collagen fibrils with distinct banding and adequate interfibrillar areas as seen in Plates 12 and 13. Although there is no evidence of the filamentous net-like arrangement as seen in micrographs of sections from specimens stained with ruthenium red without prior enzyme incubation, there is evidence in these two micrographs of fine and coarse filaments with diameters of approximately 50 Å and 125 Å respectively. A few examples of the fine filaments (arrows) still have the appearance of passing between neighbouring collagen fibrils, thus suggesting that they link the fibrils together. However, the majority of these filaments, both thick and thin, have an incomplete appearance in that they do not extend across the interfibrillar spaces from one collagen fibril to another. Therefore, these filaments, according to

diameter, have been termed fine residual filaments (FRF) and coarse residual filaments (CRF). In addition, there is still evidence of spherical masses comparable in size to those seen in Plates 4, 5 and 7; however, the distribution is more random, suggesting a slight decrease in numbers. Likewise, dense coats are also readily apparent with an average thickness of 100 Å bordering the longitudinally sectioned collagen fibrils, which is slightly less than the 150 Å average thickness of dense coats seen in micrographs of collagen fibrils in longitudinal section from specimens not incubated in papain before ruthenium red staining.

Due to the use of normal saline as the vehicle for the enzymes as employed by Myers et al. (1969), controls of additional tissue specimens were incubated in normal saline only, before fixation in ruthenium red containing glutaraldehyde and osmium tetroxide. Micrographs taken of these sections (Plates 14, 15 and 16) display examples of fine filaments only in Plates 14 and 15. Those filaments that are present are not very numerous, nor do they exhibit a high degree of electron density. However, the number of fine filaments present in Plates 14 and 15 are greater in number than those seen after incubation in bovine testicular hyaluronidase. Furthermore, even though the filaments lack ideal contrast, it is still possible to determine that, unlike the majority of filaments seen after papain incubation, these filaments after saline incubation have a consistent diameter of approximately 50 Å and none appear to have a residual character as all seem to extend from one collagen fibril to another. In addition, like the hyaluronidase and papain incubated specimens, the anastomosing of fine filaments into a net-like pattern is absent. Although none of the collagen fibrils in longitudinal section show evidence of spherical

masses, the collagen fibrils cut transversely as seen in Plate 15 exhibit peripherally located spherical masses. Dense coats are evident bordering longitudinally sectioned collagen fibrils cut in transverse section (Plates 14 and 15). The dense coats (arrowheads) associated with the collagen fibrils cut in both longitudinal and transverse section vary in thickness from 100 - 185 Å, which is comparable to the dense coats seen in micrographs of sections from tissue specimens prepared by fixation in ruthenium red containing glutaraldehyde and osmium tetroxide, without enzyme or saline incubation.

The results observed in thin sections of rat gingival periodontal ligament tissue after treatment of tissue specimen blocks with testicular hyaluronidase, papain and saline, followed by exposure to the two ruthenium red containing fixatives, are summarized in Table 4 as: no effect, partial removal and complete removal.

Table 4. Effects of Treatment with Hyaluronidase, Papain and Saline

| Electron Dense Structure | Observations Noted Following Hyaluronidase | Papain | Saline | Normals[a] |
|---|---|---|---|---|
| Fine Filaments | + + + | + + (R.F.) | + + | 20-50 Å diameter |
| F. F. - Nets | + + + | + + + | + + + | 30-45 Å diameter |
| Spherical Masses | + (160x110 Å)[b] | + ? | - | 200 Å (Length) x 190 Å (Width) |
| Dense Coats | + (65 - 100 Å)[c] | +(65 - 130 Å)[d] | - | 90-175 Å thick[e] |

KEY: -, no effect; +, mild removal; + +, moderate removal; + + +, complete removal.

R.F., residual filaments varying in diameter from fine (50 Å) to coarse (125 Å).

a & b, averages calculated from measurements of six structures each.

c & e, averages calculated from transverse sections of collagen.

d, average calculated from dense coats seen on four different longitudinal sections of collagen.

Plate 1. Collagen fibrils of normal (free of disease) rat gingival periodontal ligament perfused with ruthenium red and glutaraldehyde. Post-fixed in osmium tetroxide containing ruthenium red and embedded in araldite. No counter stain employed. Minimal electron dense structures apparent except for round deposits (arrows) along periphery of collagen fibrils. These have been termed spherical masses (SM). x 93,800.

Plate 2. Different area of the same section as Plate 1 after counter staining with uranyl acetate and lead citrate. Collagen fibrils more readily apparent but there is a masking of peripherally located electron density formerly seen without counter staining. Only evidence of electron density noted is the occasional fine filaments (FF) seen within interfibrillar areas. x 92,350.

Plate 1

Plate 2

Plate 3. Collagen fibrils (C) of normal rat gingival periodontal ligament fixed and stained by immersing the specimen block in glutaraldehyde and ruthenium red. Collagen is negatively stained and is highlighted by surrounding dense coats (DC) of ruthenium red positive material. Fine filaments (FF), also of ruthenium red positive material, project laterally from collagen fibrils and appear to anastomose (a) in interfibrillar areas. Occasionally seen at point of union of fine filaments are amorphous masses (AM). Specimen block fixed in glutaraldehyde and osmium tetroxide, both containing ruthenium red; embedded in araldite; no counter stain used. Micrograph taken of an area located very close to apical portion of epithelial attachment. Specimen A3, x 65,750.

Plate 4. Normal rat gingival periodontal collagen fibrils fixed and stained in glutaraldehyde containing ruthenium red. Further example of fine filaments (FF arrows) which appear to link one collagen fibril to another. Some fine filaments exhibit branching (b). Also seen are spherical masses (small arrows) in close association with the darker polar region of the major period bands of collagen fibrils. In addition, a fine filament appears to be linked to a collagen fibril by one of the spherical masses (arrow heads). Specimen A2, x 87,900.

Plate 3

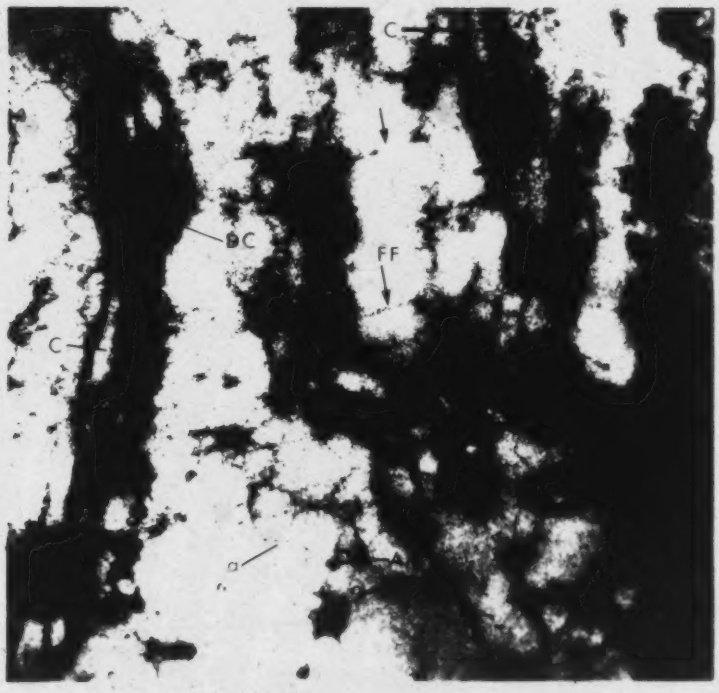



Plate 4

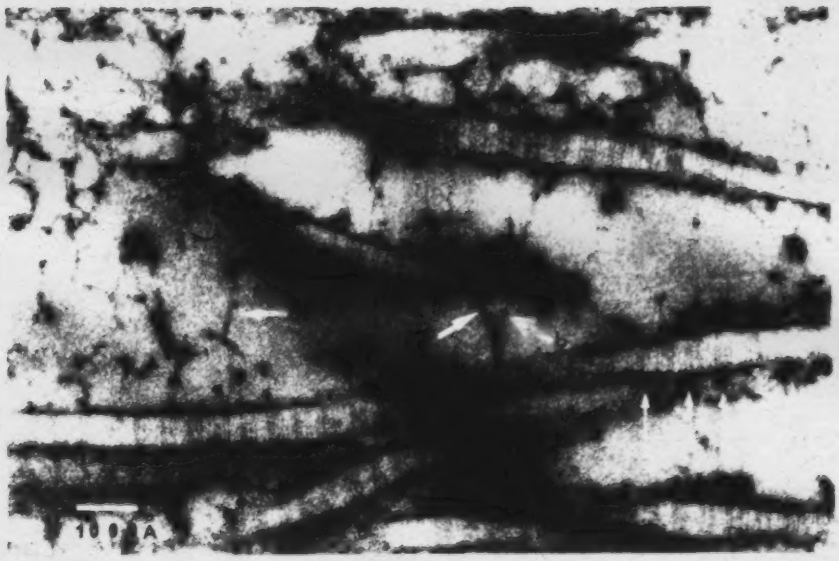


Plate 5. Collagen fibrils of normal rat periodontal ligament exhibiting further examples of electron dense material consisting of very fine filaments (FF) and spherical masses (SM). On the right middle side of the micrograph, the fine filaments exhibit anastomosing (a) and appear to be attached to collagen fibrils through a union (u) with spherical masses. Where major periodic bands of the collagen fibrils are clearly evident, the spherical masses as in Plate 4 appear to be located at the major band region (broken lines). Preparation method and location of collagen fibrils same as Plates 3 and 4. Specimen A3, x 99,500.

Plate 6. Transverse section of gingival periodontal ligament collagen fibrils. No additional staining was employed other than the ruthenium red combined with the fixative agents. Micrograph was taken of an area toward the mid-point of the trapezoid-shaped section. Note dense coats surrounding the collagen fibrils (arrows). Specimen A4, x 114,000.

Plate 5

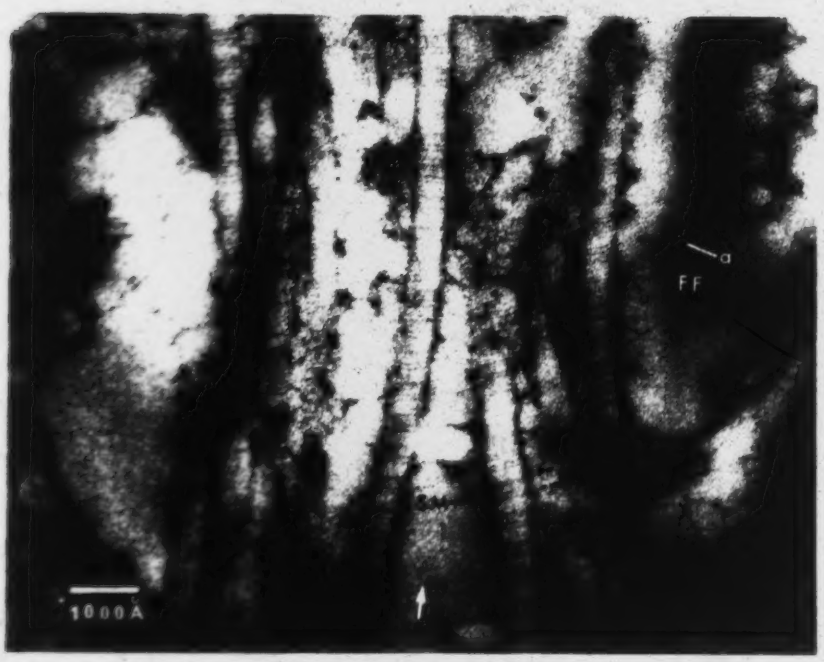



Plate 6

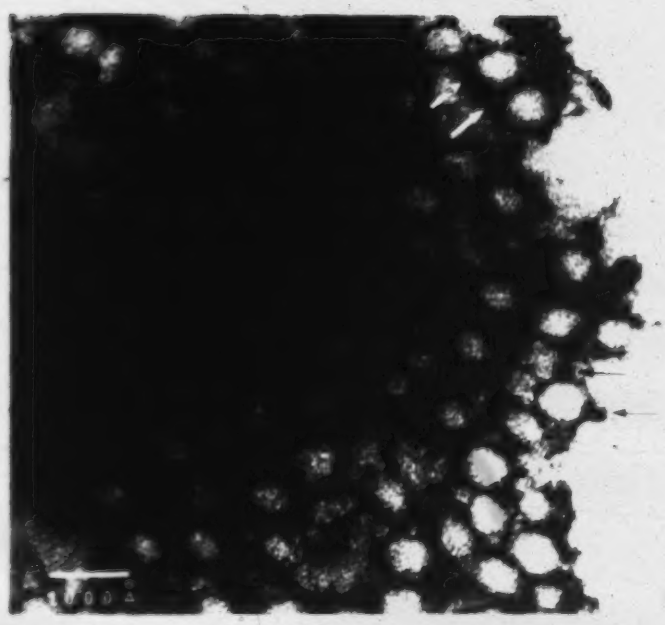


Plate 7. Additional examples of electron dense structures closely associated with collagen fibrils of the rat gingival periodontal ligament. A spherical mass (SM) is seen as a distinct circular area resembling those in Plate 4. In addition, there is evidence of fine filaments (FF arrows) and dense coats (DC). However, the electron density is not distinct in these regions, presumably due to poor penetration of ruthenium red as this micrograph was taken of an area toward the mid-point of the trapezoid-shaped section which was obtained from well within the tissue block. Specimen A3, x 99,500.

Plate 8. Interfibrillar dense coats of ruthenium red positive material. The dense coats (DC) appear to interconnect neighbouring collagen fibrils which, in their parallel alignment, are orientated with minor banding sites in register. Specimen A2, x 102,600.

Plate 7

Plate 8

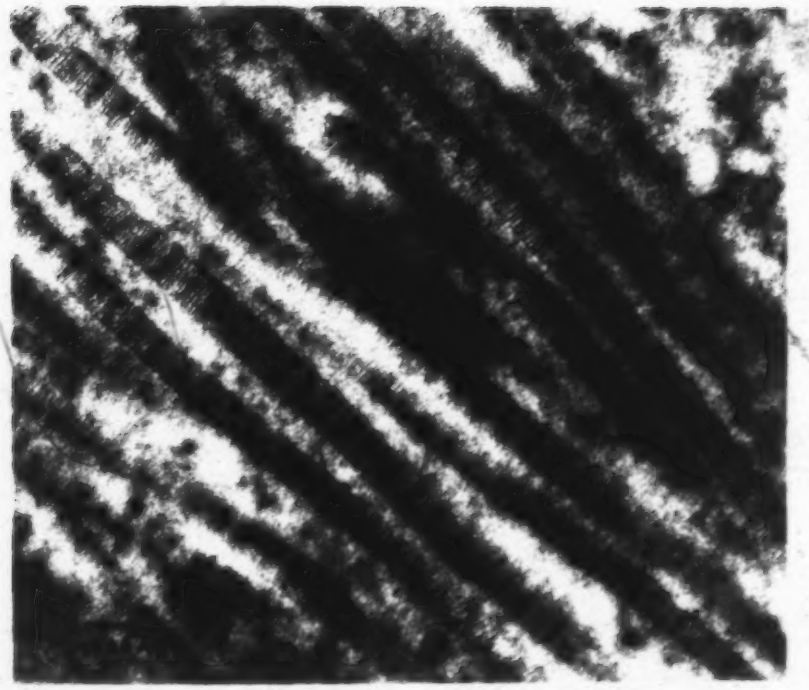


Plate 9. Collagen fibrils of normal gingival periodontal ligament. Fixed in glutaraldehyde and osmium tetroxide without the addition of ruthenium red; embedded in araldite. Section was stained with 2 percent phosphotungstic acid (pH 7.0). Note distinct minor banding of collagen fibrils, its sharp edges and absence of definite interfibrillar electron dense structures. Specimen B2, x 79,360

Plate 10. Normal gingival periodontal ligament tissue incubated in bovine hyaluronidase for one hour followed by fixation with glutaraldehyde and osmium tetroxide, both containing ruthenium red. There is no evidence of fine filaments. The only evidence of a filamentous structure is seen in the lower left region of the micrograph. These have been termed coarse filaments (CF) because of their rather thick diameter compared to the diameter of fine filaments seen in previous micrographs. There is, however, still evidence of spherical masses (SM) and a very thin dense coat (DC) bordering the longitudinal sectioned collagen fibrils. Specimen D2, x 92,340.

Plate 11. A second example of bovine hyaluronidase incubation of gingival periodontal ligament tissue. Transverse sections of collagen show only two examples of fine filaments (arrows), but clear evidence that dense coats (arrow heads) still persist after hyaluronidase incubation. Specimen D4, x 92,340.

Plate 10

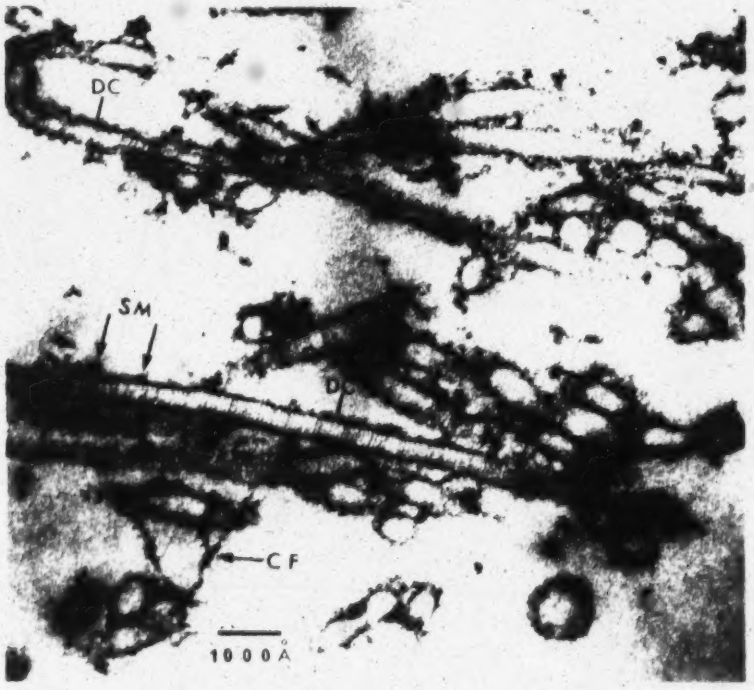
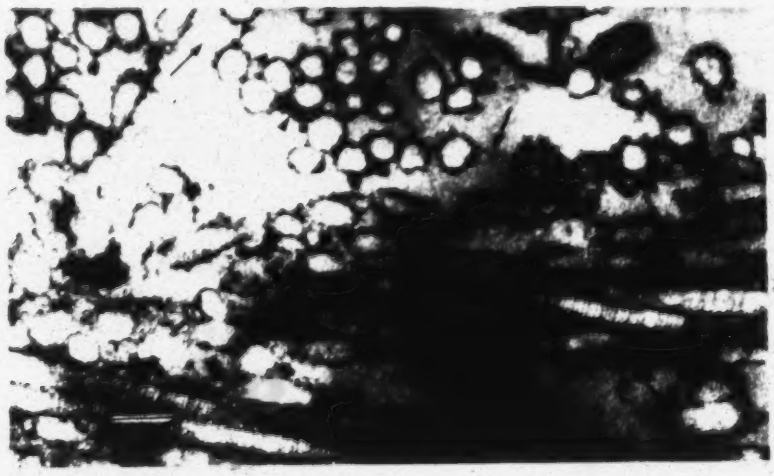



Plate 11



Plates 12 and 13. Gingival periodontal ligament tissue incubated in papain for thirty minutes, followed by fixation in glutaraldehyde and osmium tetroxide, both containing ruthenium red. Collagen banding is distinct. Filamentous structures of a residual nature are seen varying in diameter from the fine residual filaments (FRF) to coarse residual filaments (CRF). In addition, some fine filaments (arrows) still give the appearance of linking collagen fibrils together. Spherical masses (SM) and a very thin dense coat (DC) associated with the longitudinal sectioned fibrils are still evident. Plate 12, specimen C3, x 78,000; Plate 13, Specimen C1, x 78,000.

Plate 12

Plate 13

Plates 14 - 16. Gingival periodontal ligament tissue incubated in saline for one hour, followed by fixation in glutaraldehyde and osmium tetroxide, both containing ruthenium red. Only slight evidence of fine filaments (FF) is seen in Plates 14 and 15, whereas Plate 16 does not display any filaments. Dense coats (DC) are particularly obvious surrounding the transverse sectioned collagen microfibrils. Although the characteristic banding is clearly seen on the longitudinally sectioned collagen microfibrils, none of these microfibrils demonstrates the presence of spherical masses at major banding sites. However, Plate 15 does exhibit a few examples of spherical masses (SM) on the periphery of collagen microfibrils cut in cross section. Plate 14, specimen E4, x 62,525; Plate 15, specimen E4, x 80,700; Plate 16, specimen E3, x 88,000.

Plate 14

Plate 15

Plate 16

## Chapter 5

## DISCUSSION

The ruthenium red cation with its two oxo-bridges is assumed to have a linear configuration of Ru-O-Ru-O-Ru and a net valence of six cations (Fletcher et al., 1961). Crystallography studies (Sterling, 1970) indicated that the optical properties contributed by ruthenium red are due to a staining group located between the two oxygen atoms with a composition of one Ru atom and two diametrically attached $OH^-$ ions situated 4.2 Å apart. Therefore, specificity of staining is suggested as being steriospecific with any acceptor substance having anionic charges positioned approximately 4.2 Å apart, which can be substituted for the $OH^-$ ions of the staining group and thus result in an electrostatic linkage.

Schubert and Hamerman (1968) reported that the anionic groups of chondroitin sulfate are 5 Å apart in each disaccharide repeating unit and the carboxyl anion of one disaccharide unit of hyaluronic acid is 10 Å from the carboxyl anion of the neighbouring disaccharide in the polymer chain. The chondroitin sulfate negative charges are of a distance which is close enough to the requirement set by Sterling (1970) for a binding site for the ruthenium red staining group. However, the separation of negative charges positioned on the HA chain is well beyond this required distance, unless the hydroxyl on $C_6$ of N-acetyl glucosamine could serve as one of the binding site pair similar to one of the hydroxyls on the galacturonide moiety of pectic acid as postulated by

Sterling.

Further evidence of ruthenium red specificity was demonstrated in vitro by precipitate formation within solutions of ruthenium red and purified samples of hyaluronic acid, chondroitin sulfate and heparin, but not glycoprotein or sialic acid (Luft, 1971a). In addition, several other test substances also formed precipitates with ruthenium red but, according to Luft, the likelihood of encountering these in interfibrillar spaces is low unless leakage of cell contents occurs.

The above gives an explanation for ruthenium red staining of glycosaminoglycans for histochemical microscopic study. However, unless ruthenium red is accompanied by osmium tetroxide in the tissue, no density is observed in the electron microscope (Luft, 1964, 1965). It has been suggested (Luft, 1971a) that the electron density is due to the oxidation of the glycosaminoglycan with an equivalent reduction of osmium tetroxide to a lower insoluble product at the site of ruthenium red ionic binding. It was also postulated that these reactions are accomplished by a cyclic oxidation-reduction action back and forth between ruthenium red and ruthenium brown which functions as an intermediary in either a catalytic or a self-propagating mechansim. Although these later mechanisms are hypothetical, it is evident that mass density is directly proportional to ruthenium red's depth of penetration, which is 50 - 100 microns, depending on the type of tissue (Highton et al,, 1968; Luft, 1971b).

It is obvious that this rather shallow penetration of ruthenium red seen when a conventional diffusion method is employed would limit the study of a tissue specimen block to the peripheral areas. Therefore, it was assumed that vascular perfusion of ruthenium red as described in Chapter 3 would overcome its irregular and restricted diffusion into

tissues and thus yield greatly enhanced electron density in thin sections cut from any point of the specimen block. However, although the gingival tissue turned a uniform red shortly after commencement of the flow of the perfusion fluid, thus suggesting complete penetration of ruthenium red, it is evident (Plate 1) that sections taken from a depth near the mid-point of the embedded tissue specimen do not demonstrate very strong electron density. Hence, this method appears to offer little advantage for increasing electron density deep within tissue specimens of gingival periodontal ligament selected for study in this investigation.

Assuming that the red colour of the gingival tissue does indicate that ruthenium red is uniformly bound throughout the entire tissue, Luft (1971a) offers an explanation for this weak density seen within the center of tissue specimens following the perfusion method. He suggests that the meager density is a result of the rapid oxidation and inactivation of the bound ruthenium red by osmium tetroxide when the tissue blocks are placed in the ruthenium red/osmium tetroxide fixative. This reaction is made possible by the ability of osmium tetroxide to penetrate the block completely; but the ruthenium red of this solution, because of its poorer penetrating property, would not accompany the osmium tetroxide into the center of the tissue block.

Luft further suggests that this weak density within the center of the specimen block supports the "self-propagating" hypothesis rather than the "catalytic" hypothesis. That is, if the "catalytic" model was a functional reality, mass density produced in the region of the bound ruthenium red delivered by perfusion should be as intense within the middle of the specimen as it is in the peripheral areas due to the transfer of many electrons from the glycosaminoglycan substrate by one ruthenium red molecule to several molecules of osmium tetroxide.

However, as this is not the situation, it is suggested by Luft that in the absence of more ruthenium red to bind to newly generated carboxyl groups and thus propagate the reaction, an intense build-up of electron dense deposits does not occur within the center of the tissue blocks.

Although observations (Plate 1) of weak density within the middle of specimen blocks of perfused gingival periodontal ligament could suggest some support for the "self-propagating" hypothesis (Luft, 1971a), it was not possible to obtain sections from the peripheral 50 - 100 microns for comparison of density. This is due to considerable trimming from the external surface of the tissue block during removal of tooth and bone before thin sectioning could commence. Therefore, it is not known if density in the outer area of the tissue block was greater than that seen in the middle of the tissue block. Hence, without collaborative evidence of the depth of penetration of ruthenium red that occurred during post-fixation with ruthenium red and osmium tetroxide, reservations must be harboured. It could be suggested that the weak density seen was due to carrying out the post-fixation at $4^{\circ}$ C which, according to Luft (1971a), retards the formation of the electron dense reaction product compared to that formed at room temperature over a similar length of time. However, excised specimens of fresh gingival tissue that were also prepared at $4^{\circ}$ C by immersing in the ruthenium red containing fixatives did not demonstrate weak density in sections taken from the periphery of the tissue block. A reasonable explanation for this may be the concentration of diffused ruthenium red which was double that used in the perfusion solution (3000 ppm compared to 1500 ppm). This higher concentration could possibly overcome the retarded formation of reaction products resulting from the use of weak solutions of ruthenium red at low temperatures. However, Myers et al. (1969), using a 1500 ppm

concentration of ruthenium red at 4° C, obtained satisfactory density in tissue specimens from synovial membrane.

Of further interest is the study by Fowler (1970) of rat kidney specimens prepared by a similar cardiac puncture perfusion method, followed by post-fixation in ruthenium red and osmium tetroxide at room temperature, except that the concentration of the ruthenium red in the perfusion fluid was much higher (4000 ppm) and the post-fixative concentration of ruthenium red was much lower (250 ppm) than that used in this present study of gingival periodontal ligament. In his results, he reports weak to almost complete absence of electron density as well as intense deposits all within the same glomerulus. Fowler attributed the irregularity of density to the varying concentration of ruthenium red attained as a result of the extent of perfusion. Therefore, although the concentration of ruthenium red in the perfusion fluid might be of prime importance, it is suggested here that the temperature at which the post-fixative is employed does not seem to have any significance. In addition, as tempting as it is to accept the "self-propagating" hypothesis of Luft (1971a) for the explanation of weak density as a result of perfusion, the concept must remain theoretical until extensive investigations provide further evidence of the actual reaction between ruthenium red and osmium tetroxide, as well as the source of electrons from the glycosaminoglycans that are required for the reduction action. Furthermore, in order to properly evaluate fibrous tissues prepared by vascular perfusion with ruthenium red, carefully controlled studies should be done using varying concentrations of a standard commercial preparation of the dye in both the perfusion fluid and the post-fixative solution at 0° C and room temperature.

Perhaps such a study could employ rat gingival periodontal ligament tissue as before, except after perfusion the supra-alveolar tissue should be removed by the gingivectomy technique before post-fixation in osmium tetroxide and ruthenium red. This would allow the transverse cut periodontal fibers which formerly inserted into the cementum to be freely exposed to ruthenium red of the post-fixative solution, thus adding to the ruthenium red delivered by perfusion.

However, as reported in the results of this present investigation, thin sections from excised fresh gingival tissue fixed and stained by the sequential immersion in the two ruthenium red containing fixatives did demonstrate adequate electron density for study, provided that the area examined was confined to the outer 50 - 100 microns of the specimen block which was proximal to the cementum surface before excision. These electron dense deposits were seen as four forms; interconnecting fine filaments extending at approximate right angles between collagen fibrils, a net-like arrangement of fine filaments in interfibrillar spaces, spherical masses apparently situated at specific sites within the major band repeating period, and dense coats surrounding collagen fibrils.

Other studies (Haust, 1965; Hashimoto, 1967; Griffin and Harris, 1967) have also described filamentous structures varying in diameter from 40 - 150 Å within interfibrillar areas of connective tissue following staining of thin sections with uranyl acetate only or in combination with lead citrate. These filaments or "microfibrils", as they were termed, demonstrated in most instances a periodicity or beaded appearance and were considered to be of a protein nature representing either precursor elements of collagen fibrils (Hashimoto, 1967) or collagen and elastin fibrils (Haust, 1965) or the protein moiety of a protein-polysaccharide complex (Griffin and Harris, 1967). An amorphous

component seen with the beaded elements was interpreted by Griffin and Harris as being the polysaccharide moiety. Similarly, amorphous material located between collagen fibrils of the rat gingival periodontal ligament (Plate 9) is also noted following staining of thin sections with PTA (pH 7.4) which, at this pH, is a general protein stain (Pease and Bouteille, 1971). However, it is possible that this is a result of background staining of the embedding material. Nevertheless, it is significant that with PTA staining no electron dense morphology is noted similar in appearance to the fine filaments seen after ruthenium red staining as this would indicate a composition of only protein. The negative staining of collagen fibrils also indicates that ruthenium red is not a protein stain. The question could arise that collagen microfibrils of the same diameters as ruthenium red positive filaments (30 - 50 Å) might have reactive staining sites for ruthenium red. However, ruthenium red was shown to be non-reactive to several test proteins (Luft, 1971a). Furthermore, it is obvious from the results produced by block staining with ruthenium red that the fine filaments seen do not have a beaded appparance; nor, except for the coarse residual filaments seen after papain incubation, do these filaments exceed a diameter of 50 Angstroms. These coarse filaments (125 Å in diameter) seen after papain, when examined closely (Plates 12 and 13), appear as if they are displaced electron dense elements as they lie on top of and not between adjacent collagen fibrils as do the fine filaments.

Therefore, it is suggested that these electron dense structures are highly likely the glycosaminoglycan moieties of proteoglycans, as indicated by their removal in whole or in part following incubation in testicular hyaluronidase, papain and saline (Table 4, page 95). Biochemical analyses of rat periodontal ligament (Kofoed and Bozzini, 1970)

have confirmed the presence of dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and hyaluronic acid. In addition, radioautographic studies with injected $S^{35}$ have previously demonstrated that sulfated glycosaminoglycans are synthesized in the fibroblast and appear in the amorphous ground substance of mouse periodontal ligament (Baumhammers and Stallard, 1968). However, there have been no previous reports of glycosaminoglycans being closely associated with collagen fibrils of rat gingival periodontal ligament at the electron microscope level, as was found in this investigation.

The results indicate that the fine filaments extending laterally from collagen fibrils are composed of hyaluronic acid and/or chondroitin sulfates as these electron dense structures were digested almost entirely by testicular hyaluronidase and partially by papain, thus also suggesting a protein component. The limited number of fine filaments remaining after incubation in normal saline also suggests the presence of hyaluronic acid as 0.17 M Na Cl as well as water will extract this polysaccharide from connective tissue homogenates (Hallén, 1970; Toole and Lowther, 1966). Hence, the complete removal by saline of the net-like arrangement of fine filaments seen in Plates 3 and 5 indicates that these structures are probably composed entirely of hyaluronic acid. Likewise, the spherical masses appear to be definitely affected in number and possibly size by prior incubation in hyaluronidase with similar but lesser effects following papain treatment. However, as was pointed out in the results (Table 4, page 95), it is not certain that papain had a measurable effect upon the spherical masses, except for the more random distribution of these structures seen in Plates 12 and 13 when compared to the more evenly distributed spherical masses seen in Plate 4. This finding is difficult to explain in view of the removal of the spherical

masses by hyaluronidase. The only explanation that can be offered is that the spherical masses are a proteoglycan with a low protein content such as hyaluronic acid, which is known to be associated with relatively little protein (Sandson and Hamerman, 1962; Silpananta et al., 1968).

The structure most closely associated with the gingival periodontal collagen fibrils, the dense coats, were only slightly reduced in thickness by the action of testicular hyaluronidase. It is assumed that this indicates a heterogeneous composition of dermatan sulfate and minor amounts of chondroitin sulfate, as reported by Fransson and Rodén (1967a, 1967b) and Fransson (1968). A slight diminution in thickness of the dense coats is also noted after papain treatment, again indicating the presence of a protein component as would be found in proteodermatan sulfate. Similar dense coats surrounding collagen fibrils of different fibrous connective tissues have been reported after tissue block exposure to ruthenium red and osmium tetroxide (Luft, 1964, 1965; Highton et al., 1968) and reduced thickness of coats by testicular hyaluronidase and papain treatment followed by ruthenium red and osmium tetroxide (Myers et al., 1969).

Toole and Lowther (1966) found that dermatan sulfate released from bovine skin by proteolytic digestion contained amino acids even after extensive purification, which suggests that dermatan sulfate is tightly bound in the native state to a protein presumed to be collagen. The resistance of the dense coats to papain digestion in this study of gingival periodontal ligament and the finding of Toole and Lowther would suggest that the dense coats are composed mainly of proteodermatan sulfate.

The peripheral location of the spherical masses at a specific site along the major repeating period of the collagen fibril and its

observed morphology is supported by Nakoe and Bashey (1972) in their study of rat tail tendon and bovine heart valves exposed to ruthenium red and osmium tetroxide. These workers, with the aid of high magnification micrographs, determined that a continuous row of these perifibrillar structures were situated at the same minor band site in each major band period, resulting in a regular 640 Å center-to-center distance.

The apparent linkage of the fine filaments, either directly or indirectly, by spherical masses (Plate 5) to the darker staining portion of the major banding of neighbouring collagen fibrils is also supported by the micrographs of Nakoe and Bashey (1972) which show a fine filament interconnecting transverse sectioned collagen fibrils and interfibrillar filaments apparently associated with the perifibrillar round masses located on the longitudinal sectioned collagen fibrils. In agreement with the findings in the present study, fine filaments of a similar size (30 - 35 Å) have been demonstrated in human knee synovial membrane collagen fibrils after staining and fixing in the two ruthenium red containing fixatives (Myers et al., 1969). In addition, these fine filaments seen in synovial membrane were highly orientated in respect to the major banding of collagen fibrils and appear to form bridges between adjacent fibrils. These workers also reported electron dense structures termed "transverse belts" overlying the periodic banding and projecting outward from the edge of the collagen fibrils approximately 200 Angstroms. The results noted after testicular hyaluronidase indicated a composition of hyaluronic acid and chondroitin sulfate. Therefore, it is reasonable to assume that the transverse belts, because of their position and composition, are similar in nature to the perifibrillar round masses of rat tail tendon collagen fibrils (Nakoe and Bashey, 1972) and the spherical masses of rat gingival periodontal ligament collagen fibrils.

However, because of the slight difference in morphology, it is suggested that this represents a species variance in glycosaminoglycans. A similar suggestion of specie variation could be made following comparison of the fine filament networks seen in gingival periodontal interfibrillar areas and the intermediate fibrils seen in synovial interfibrillar areas (Myers et al., 1969) as both of these structures appear to consist mainly of hyaluronic acid.

The hyaluronic molecule has a shape in the configuration of a flexible, randomly coiled, long chain polymer occupying a spherical domain as indicated by data from sedimentation and streaming birefringence measurements (Ogston and Stanier, 1951, 1953) as well as light scattering measurements (Laurent and Gergely, 1955; Rowen et al., 1956). The intermediate fibrils of synovial membrane reported by Myers et al. (1969) do exhibit some coiling and therefore resemble this description of the hyaluronate molecule. Along these lines, it is tempting to postulate that the net-like arrangement of fine filaments seen between gingival periodontal ligament collagen fibrils represents a different type of model structure for the hyaluronate molecule. The finest elements, the filaments measuring 30 - 45 Å in diameter, represent the polysaccharide component and the amorphous masses seen occasionally at the points of union of the fine filaments are possibly minor amounts of protein. A structure of this nature in its three-dimensional form could serve well in a role of resisting compression as suggested by Fessler (1960).

However, there are factors that make acceptance of this alternate model of the hyaluronate molecule untenable. Firstly, the openings of the mesh range from 500 - 1000 Å and even larger in some areas, which would allow passage of very large particles. This is in contradiction

to the study by Laurent and Pietruszkiewicz (1961) who noted that the retarding effect of the hyaluronate macromolecular structure was greatest against particles over 400 Angstroms. Secondly, although there was a decrease in ruthenium red staining structures noted in this investigation after incubation in papain, this is interpreted as a loss of stainable glycosaminoglycan moieties due to removal of segments of the protein moiety to which the glycosaminoglycan chains are covalently bound. In addition, there is at present no evidence to support an in vivo reaction between ruthenium red and proteins in the native state. Furthermore, in vitro experiments indicate that ruthenium red is unreactive with several test proteins (Luft, 1971a).

It has been postulated that proteoglycan macromolecules have a functional role of orientation and stabilization of collagen microfibrils (Mathews, 1965; Luft, 1971b; Jackson and Bentley, 1968). In regard to the dense coats surrounding collagen fibrils, it is suggested (Luft, 1971b) that they provide orientation by promoting registration of minor banding between neighbouring fibrils. It was further suggested that tendon with its dense packing of collagen fibrils may derive strength from this alignment of fibrils. Supportive evidence for an orientation function by interfibrillar dense coats is indicated by the parallel array of gingival periodontal collagen fibrils seen in Plate 8. In this micrograph neighbouring collagen fibrils separated by electron dense interfibrillar material appear to be orientated with similar minor bands in register. Such an alignment of gingival collagen fibrils, if created for maximum strength, would be highly beneficial in resisting forces applied to the supra-alveolar tissues during mastication. Additional support for the concept of proteoglycan stabilization of collagen microfibrils is suggested by the perpendicular orientated fine filaments as the

majority of these filaments seen appear to link together adjacent periodontal collagen microfibrils. The wide interfibrillar spaces and the residual filaments remaining after incubation of tissue blocks in papain, followed by exposure to ruthenium red and osmium tetroxide, would give some supportive evidence to this concept.

Mathews (1965), from light scattering, viscosity and enzymatic techniques (Mathews and Lozaityte, 1958), has proposed a model for the interaction between collagen microfibrils and the proteochondroitin sulfate molecule (Figure 3, page 22). The fine filaments resemble the polysaccharide side chains which are covalently linked to the central protein core (Rodén and Armand, 1966) but electrostatically bound to the collagen fibrils in parallel alignment to the non-collagenous protein core. It is quite likely, but not definite, that these fine filaments are composed of chondroitin sulfate disaccharide repeating units due to their removal by testicular hyaluronidase. However, what is not obvious is the position of the central protein core because it is presumed that proteins do not stain with ruthenium red; nor has protein component of the proteochondroitin molecule been revealed by double staining of ruthenium red stained sections with heavy metals. The polypeptide chain may be situated between collagen fibrils as Mathews suggests; however, if this were the situation, one would expect to see fine filaments extending from a collagen fibril halfway across the interfibrillar spaces to its point of covalent linkage to serine (Rodén and Armand, 1966). Then, either above or below, separated by several Angstroms, a continuation of another polysaccharide chain to the collagen fibril on the opposite side should also be evident. This discontinuation of filaments extending from one collagen fibril to another was not noted in any of the micrographs included in the results of this

investigation. The positioning of a central protein core directly on the collagen fibrils also cannot be substantiated due to the residual filaments remaining after papain incubation. That is, if the portion of the filament removed was formerly attached at its disrupted end to the protein by a covalent linkage, this would suggest that the terminal end of the filament still attached to the collagen fibril would have to be linked by a stronger bond than an electrostatic force. Conversely, if the attached residual filament was due to incomplete digestion of the collagen linked protein core by papain, this would suggest that the portion removed was more than just polysaccharide in nature. The only conclusion that could be drawn is that the fine filaments represent the entire proteochondroitin sulfate molecule as suggested by Fitton Jackson (1964) with the protein core overlaid by a coiled polysaccharide chain. A third alternative model for the interaction of glycoprotein, proteoglycan and collagen fibrils, in order to produce a stabilized structure, has been proposed by Jackson and Bentley (1968). This model (Figure 4, page 22) suggests an extension of the polysaccharide side chains over several collagen fibrils. However, it is quite obvious from the micrographs of rat gingival periodontal ligament tissue that there is no evidence of the electron dense filaments superimposed on collagen fibrils.

In addition, one of these fine filaments associated perpendicular to collagen fibrils (Plate 4) suggests a branching effect. Myers et al. (1969) noted short side chains on some of the fine lateral filaments they observed. However, the branching effect seen in this present study is much more definite and appears to be linked to the collagen fibril at both terminal ends of the branch. Unfortunately, this branching was noted in only one other micrograph (Plate 10); therefore, without further study, it would be premature to attempt any interpretation of

the significance of this structure.

It is concluded from the effects of enzyme treatment and other supportive evidence offered by the results of similar studies with ruthenium red that these electron dense structures, which are seen closely associated with rat gingival periodontal collagen fibrils, are highly likely the glycosaminoglycan moiety of proteoglycans. However, it is not certain if the observations noted represent the true morphology of these structures in the native state; nor is it certain that the structures seen represent the total amounts present. Of concern is the fact that cationic dyes result in marked shrinkage of the tissue and often distortion of fine structure (Szirmai, 1963). This is countered by the effects of the aldehyde fixatives which preserve the protein moiety of the proteoglycan. However, Szirmai considers that this reaction with protein lowers the isoelectric point, thus interfering with the subsequent staining by the cationic dye. In addition, aqueous solutions of formaldehyde will extract glycosaminoglycans to a considerable extent (Szirmai, 1963). Hence, it is more than likely that glutaraldehyde used in this investigation might also remove some of the glycosaminoglycans before stabilization by the cationic ruthenium red.

It is therefore quite evident that until more sophisticated methods are developed for preservation of the glycosaminoglycan, considerable caution must be employed during the interpretation of the results. Also, a much broader range of enzymes other than testicular hyaluronidase and papain must be used for specific identification of each of the glycosaminoglycans. Furthermore, cetylpryidinium chloride should be employed as it has been shown to block the staining of ruthenium red (Gustafson and Pihl, 1967a).

When these refinements in the technique have been perfected and

when the knowledge is complete of glycosaminoglycans in normal gingival periodontal ligament, then and only then will it be possible to do comparative studies of glycosaminoglycans in the diseased state.

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# APPENDIX

Although perfusion of ruthenium red combined with glutaraldehyde may prove to be effective in overcoming the poor penetration of ruthenium red, specificity of staining of the glycosaminoglycans by ruthenium red must be established by the methylation technique and enzymatic digestion of unfixed tissue specimens. Therefore, a second method of obtaining and preparing fresh periodontal tissue was employed. This method consisted of removal of the mandibles and cutting 1/2 millimeter bucco-lingual sections with the bone and tooth sectioning machine (as described in Chapter 3) for obtaining specimens after perfusion fixation. However, instead of using the fixative glutaraldehyde as the disc and tissue coolant, cold normal saline (pH 6.2) was used. As the sections were cut, some were immediately placed in either cold (4° C) 1.2 percent glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) with 1500 ppm ruthenium red for a period of fourteen hours, or in the same solution without ruthenium red for a similar length of time.

Other sections were incubated in hyaluronidase, papain and saline, according to the method of Myers et al. (1969): that is, bovine testicular hyaluronidase (Worthington Biochemical Corporation, 1500 units/ml, in normal saline) for a period of one hour at 37° C; papain (Worthington's, 130 units/ml, in normal saline) at 37° C for thirty minutes and normal saline for one hour at 37° C. In addition, some of these specimens were methylated with 0.05 N HCl in methanol at an incubation temperature of 58° C and times of one, two and four hours

(Fisher and Lillie, 1954). Following incubation of the tissues in methanol HCl, enzymes and normal saline, the specimens were briefly rinsed in cool ($10^{\circ}$ - $15^{\circ}$ C) clear 0.1 M Na cacodylate buffer and fixed for fourteen hours at $4^{\circ}$ C in the 1.2 percent glutaraldehyde, sodium cacodylate and 1500 ppm ruthenium red solution. Then all the specimens, except those controls fixed in glutaraldehyde without ruthenium red, were given three twenty minute rinses in cold ($4^{\circ}$ C) 0.1 M Na cacodylate buffer (pH 7.4) with 800 ppm ruthenium red before post-fixation in 2 percent osmium tetroxide in 0.05 M Na cacodylate buffer with 400 ppm ruthenium red, for a period of four hours at $4^{\circ}$ C. The glutaraldehyde without ruthenium red fixed specimens were treated in the same manner, except that ruthenium red was omitted from all solutions. From this point onward, the dehydration, clearing, embedding in araldite and preparation of cured specimen blocks for sectioning was of the same procedures as that described for the perfusion fixed tissue specimens.

Sections 1 micron in thickness were cut from sample tissue specimens selected from the ruthenium red treated tissues and the enzyme incubated tissues. These sections were stained with toluidine blue and examined with the light microscope. It was noted that the epithelial cells of the attached and free gingiva demonstrated marked intracellular edema, and the collagen fibers did not appear to stain as intensely as the sections from perfusion fixed specimens. Thin sections for electron microscope study did not cut well and, when examined in the electron microscope, the sections exhibited torn areas and the collagen fibrils were of a very low density which necessitated counter staining with heavy metals.

One micron sections from the methylated tissues were also stained with toluidine blue and examined in the light microscope. These sections

exhibited considerable disturbance in morphology which appeared as loss of cellular structure and large interfibrillar spaces. The degree of disturbance increased with the incubation time; therefore, only the tissue specimens methylated for one hour were selected for electron microscope study. Thin sections, when examined in the electron microscope, did not demonstrate normal architecture with regard to the collagen fibrils. The collagen fibrils were "bunched" together, leaving wide artifact spaces between the groups of fibrils.

Revel (1964) also noted that electron microscopic specimens are fairly badly damaged by methylation in 0.1 N HCl at 60° C for twenty minutes. However, he claimed that it was possible to determine abolition of colloidal thorium stainable material in areas where ultrastructural detail was retained. Although in this present study of gingival periodontal ligament it too appeared that there was no readily observable electron density, thus indicating inhibition of the uptake of the cation dye by the tissue anions, this could not be demonstrated conclusively in the interfibrillar areas due to the closely packed collagen fibrils. Hence, no micrographs were taken for inclusion in the results of this thesis.

In addition, because of the unfavourable results with the tissues obtained by bucco-lingual discing of the rat mandibles, gingival tissues obtained by the gingivectomy technique were not subjected to methylation.

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